Xanthomonas blight of cassava: studies on bacterial pathogenicity and host resistance in vitro and in planta

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XANTHOMONAS BLIGHT OF CASSAVA: STUDIES ON BACTERIAL PATHOGENICITY AND HOST RESISTANCE IN VITRO AND IN PLANTA

Submitted by Nelum Deshappriya for the degree of Ph.D. of the University of Bath.

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TO WASANTHA AND RAMINDU WITH LOVE.

ABSTRACT

A study of the interaction between Xanthomonas campestris pv manihotis (Xcm) and cassava plants was carried out using tissue culture systems and whole plants. Of the tissue culture systems developed and tested, plantlets and suspension cultured cells of cassava proved to be suitable to study the susceptible response. Plantlets showed symptoms similar to those of whole plants. Xcm caused death of suspension cultured cells and the rate of cell killing increased with plant cell age. No cell death occurred when the bacterial and host cells were separated by dialysis membrane indicating either the necessity of contact between host and parasite or the involvement of a high molecular weight factor produced by Xcm to mediate plant cell killing.

Field resistant cassava cultivars failed to show hypersensitivity or a high level of resistance when challenged by the bacterium. However, disease development in leaves and stems of resistant cultivars was delayed (by 2-3 days) as compared to appearance of symptoms in susceptible cultivars. A quantitative study of bacterial multiplication in cassava leaves showed that growth in the resistant cultivar was slightly less but not significantly different than in the susceptible cultivar. Electrolyte leakage from leaf discs from both resistant

and susceptible cultivars was induced by Xcm but again a delay in response was observed with discs from resistant cultivars.

The rapid spread of Xcm in cassava indicated movement in vascular tissues. This was confirmed by transmission electron microscopy (TEM) of infected cassava stems and leaves which clearly showed that Xcm cells were localized within vessels and xylem parenchyma but in stems bacteria were also present in intercellular spaces of xylem parenchyma; no other tissues were invaded.

A study of the nature of pathogenicity determinants of Xcm was conducted during bacterial growth in artificial media and also in vivo when possible. The synthesis of a toxin by Xcm was tested under a range of culture conditions but culture fluids never showed any toxicity towards cassava. However, a toxic metabolite of methionine had previously been reported to be synthesised both in vitro and in vivo. However, an analysis of free amino acids in cassava tissues obtained from healthy, and infected leaves showed the methionine levels were too low (1/25-1/250) for toxin production. However, Xcm produced low protease activity which could break down bound methionine to release levels necessary for toxin production in vivo. Also, there was no evidence by TEM for activity of a diffusible toxin in planta.

A study of cell wall degrading enzymes with a potential for involvement in pathogenicity revealed the production *in vitro* of a highly inducible polygalacturonate lyase (PGL) with a single isozyme (pI *ca*. 9.0). *Xcm* isolate 2967 produced very high PGL levels but production by other pathogenic isolates was much lower. Nevertheless, these low levels of PGL could be sufficient for successful pathogenesis because even at high dilutions PGL rapidly caused death of cassava cells. However, extracts from diseased cassava plants failed to show any PGL activity and extensive breakdown of host primary walls was not evident by TEM.

TEM revealed copious production of extracellular polysaccharide (EPS) in all cells infected with *Xcm*. It is concluded that macroscopic wilting and localized cell death result from water stress induced by bacterial EPS and by vascular responses such as vessel waterproofing.

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ABBREVIATIONS

BAP -6-Benzylaminopurine

cfu -colony forming units

cm -centimetre(s)

ca. -circa

cv. -cultivar

CWDE -cell wall-degrading enzymes

°C -degrees centigrade

d -day(s)

2,4-D-2,4-dichlorophenoxyacetic acid

E.a. -Erwinia amylovora

FDA -Fluorescien di acetate

g -gram(s)

h -hour(s)

IEF -Isoelectric focusing

kg -killogram(s)

l -litre(s)

M -Molar

m -metre(s)

min -minutes(s)

mg -milligram(s)

ml -millilitre(s)

µl -microlitre(s)

µE -micro Einstein

Mm -minimal medium

mm -millimetre(s)

MS -Murashige and Skoog basal medium

MS_{20} -Murashige and Skoog medium with 20g/l sucrose

NaPP -sodium polypectate

nm -nanometres

NYGA -nutrient yeast glycerol agar

NYGB -nutrient yeast glycerol broth

OD -optical density

PG -Polygalacturonase

PGL -Polygalacturonate lyase

PME -Pectinmethyl esterase

PL -Pectin lyase

pv -pathovar

% -percentage

rpm -revolutions per minute

RUV -relative viscometric units

sec -seconds

SDW -sterile distilled water

TEM -transmission electron microscopy

ul -microlitre(s)

v -volume(s)

w -weight

Xcc -Xanthomonas campestris pv campestris

Xcv -Xanthomonas campestris pv vesicatoria

Xcm -Xanthomonas campestris pv manihotis

CONTENTS

INTRODUCTION

Cassava, Manihot esculenta Crantz, is a dicotyledonous plant belonging to family Euphorbiaceae, and like most other members of that family, contains laticifers and produces latex. M. esculenta has a chromosome number 2n=36 (Perry, 1943; Martin, 1976).

Cassava is a monoecious species and is highly heterozygous. It is protogynous, the female flowers opening about 10 days earlier than the male flowers and this favours outcrossing. Cassava is propagated vegetatively by stem cuttings. In nature and during the process of plant breeding, propagation by seed occurs although plants are slower in establishment (Williams and Ghazali, 1969).

Stem cuttings produce an extensive, fibrous, adventitious root system and the tuberization process involves the secondary thickening in some of these roots. Tuber formation commences by the 8th week after planting if the environmental conditions are favourable.

The cassava plant grows as a shrub with the stem reaching heights of 1-4 metres depending on the variety. The internal structure of the stem corresponds to that of a typical dicotyledon. The leaves of cassava, which have a deeply palmate lamina, are spirally arranged on raised nodal portions on the stem. The number of lamina lobes is

usually 5-7 although numbers from 3-9 may occur. The leaf of cassava is deciduous and it exists only for a few months.

All cassava plants and tubers are poisonous to a certain degree because they contain the cyanogenic glucoside linamarin which breaks down to give hydrogen cyanide. To circumvent this the tuber is normally cut into pieces and boiled before being eaten.

1. Origin and dispersal of cassava

The cassava plant is thought to have originated in northeast Brazil although central America could be an additional centre of origin (Rogers, 1963). Today, *M. esculenta* does not exist in the wild state but is cultivated in all tropical regions of the world.

Cassava had spread rapidly from South America to the west coast of Africa by the end of the sixteenth century and to the east coast by the end of the eighteenth. The crop had arrived in India in about 1800 and it had probably been introduced to Indonesia and the Phillipines from Mexico by about the same time and to Mauritius about 1740. Sri Lanka officially imported cassava cuttings from Mauritius in 1786. By the nineteenth century cassava was established as a crop in south and southeast Asia (Leon, 1977).

In the the mid nineteenth century it became widely grown as a famine reserve on account of its tolerance of drought, locust attack and poor husbandry (Jennings, 1976).

Controlled breeding such as interspecific crossing and back crossing started in Java in 1920 and in east Africa in 1937. Some of the objectives in breeding were resistance to bacterial and viral diseases, resistance to drought,improved vigour, low cyanogenic glycoside content and increased protein content (Onwueme, 1978).

2.Environmental conditions for cassava growth

Cassava is a crop of the lowland tropics, and it needs a warm, moist climate with mean temperatures of 25-29°C. Cassava needs an optimum rainfall of 100-150 cm/yr. However, the crop is well adapted to cultivation under conditions of drought as well as poor soil conditions. Cassava is most productive between latitudes 15°N and 15°S but it can be grown between 30°N and 30°S under very broad climatic and edaphic conditions (Onwueme, 1978).

3.World production and economic importance of cassava

In 1989 the world production was about 147 million tons (FAO report,1991). It is the fourth most important crop in terms of calories produced for consumption in the tropics (Cock,1985). Africa, South America and Asia account for nearly all the production of cassava. There

are an estimated 70 million people who obtain more than 500 cal/day from cassava (Cock, 1985).

Cassava tubers are processed to remove the hydrogen cyanide and eaten as gari, farinha de mandioca and retted cassava (Onwueme, 1978). Tubers are also processed and eaten as flour, starch and chips.

In nutritional terms, cassava must be considered primarily as an energy source that contributes little else, except vitamin C. The protein of cassava tuber is rich in arginine but low in methionine, lysine, tryptophane, phenyl alanine and tyrosine (Oyenuga, 1968). The composition of the tuber is given in Appendix Ⅱ.

The fresh cassava tuber can also be used as feed for livestock such as sheep, goats, cattle and pigs. Cassava leaves and tender shoots are also used for human consumption particularly in Sierra Leone and in the Congo basin (Onwueme, 1978). Cassava leaves compare with soybeans in protein quality and are higher in lysine, although methionine and possibly tryptophane are deficient.

Cassava can be used as raw material in starch production and this starch has uses in the food industry, paper making,as a lubricant in oil wells and in the textile industry and as the substrate for the production of dextrins. Alcohol production from cassava has also

been of interest, especially in Brazil (Onwueme, 1978).

4.Diseases of cassava

4a.Viral diseases

Cassava mosaic disease, common mosaic disease, brown streak disease and cassava vein mosaic disease are the reported virus diseases of cassava (Lozano and Booth, 1974). Cassava mosaic disease occurs in all parts of East, West and Central Africa and adjacent islands (Chant, 1959; Jennings, 1960) and the reported losses in yield range from 20-90% (Chant, 1959; Jennings, 1960; 1970). This disease does not appear in other countries and is absent from the Americas (Jennings, 1960). Common mosaic disease is reported to occur in various parts of Brazil and the losses in yield range from 10-20% but because of its ease of control the disease is considered comparatively unimportant (Costa et al., 1970; Nolt et al., 1992). Cassava brown streak disease is reported only to occur along the east coast of Africa and losses are difficult to estimate because the plants are usually infected simultaneously with mosaic disease (Jennings, 1972). Cassava vein mosaic disease is reported to occur in Brazil, but little reliable information is available (Costa et al., 1970).

4b. Fungal diseases

Leaf spots are reported to be caused by several Cercospora spp. (Castano,1969) and the most important is brown leaf spot caused by C. henningsii (Powell, 1972). Leaf spots caused by Phyllostica has been reported from Colombia (CIAT, 1972), Brazil (Viegas, 1943), Phillipines (Sydow, 1913) and tropical Africa (Vincens, 1915).

White thread disease caused by Fomes lignosus, cassava anthracnose and super elongation disease caused by Sphaceloma spp. are two other fungal diseases reported (CIAT,1976).

4c. Bacterial diseases

A number of bacterial diseases of cassava have been reported in different parts of cassava growing areas of the world. These are: a vascular disease caused by Pseudomonas solanacearum, a combination of leaf spot, necrosis of petioles and subsequent defoliation caused by Erwinia cassavae, a severe leaf spot caused by Xanthomonas cassavae and blight and wilting caused by Xanthomonas manihotis (Lozano & Booth, 1974).

Xanthomonas campestris pv manihotis (Xcm) -

On a global basis, cassava bacterial blight caused by Xcm is considered to have done more damage to the crop than any other disease during the past two decades.

It is now recognized as one of the most important factors limiting production in affected areas. It is reported to cause between 12-90% loss of yield in affected areas and sometimes complete destruction (Lozano,1986 ;Maraite & Meyer,1975). Planting infected cuttings can cause about a 30% loss in yield and if the environmental conditions are favourable losses can reach upto 80% in three cycles. Invasion by weak pathogens such as Colletotrichum spp. and Choanephora cucurbitarum can cause a 90% loss. An epidemic in Zaire caused a 75% loss of yield (Maraite & Meyer, 1975). During this time the losses in Central Africa were as high as 80% (Persley, 1976). In 1974, this disease reduced yields by 50% in a plantation in Brazil (Lozano, 1986).

This disease was first reported from Brazil (Bondar, 1912) and has since been reported in Argentina (Zyngier de Resnik, 1968), Colombia (Lozano, 1972 ; Lozano & Sequeira, 1974b), Nicaragua and Gautamala (Normanha, 1971), Nigeria (Williams et al., 1973), Zaire (Maraite & Meyer, 1975), Madagascar (Boriquet, 1949), Mauritius (Orieux & Felix, 1968), Thailand, Malaysia and Taiwan (Onwueme, 1978). Disease surveys conducted in Trinidad between 1985-1987 showed that cassva bacterila blight is present in all but one county of the country (Joseph & Elango, 1991).

Disease symptoms are characterised by leaf spotting

and blight, wilting of young stems and leaves, stem dieback, gum exudation and vascular necrosis. Primary symptoms resulting from planting infected material are wilting of the young germinated sprouts, followed shortly by dieback (Lozano, 1986). Symptoms resulting from secondary infections show leaf spotting followed by blight, wilt and stem dieback (Lozano & Booth, 1974). Leaf spots start as water soaked areas, small at first and angular but these enlarge to cover parts of the leaf which then turns brown (Pereira & Zagatto, 1967). These nerotic leaves dry up and eventually abscise. Gum is exuded from young infected stems, petioles and leaf spots (Bondar, 1912; Pereira & Zagatto, 1967). Gum contains bacteria which may be dispersed by rain water as a new source of infection (Lozano, 1972; Williams et al.,1973). Vascular strands of infected petioles and stems necrose and appear as brown strings. This vascular discolouration may spread into roots (Lozano,1972).

Infections destroy the aerial parts of immature plants. When this occurs, the plants produce new shoots from the stem base which are extremely disease susceptible and during rainy seasons these rapidly become infected prolonging the disease.

The pathogen is a gram negative slender rod, 1.4-2.8µx0.35-0.93µ (Burkholder, 1942), 0.76-2.69µx0.32-0.49µ (Lozano & Sequeira, 1974a), motile by means of a single

polar flagellum, not encapsulated and not spore forming (Lozano, 1972). It is aerobic, relatively fast growing and unlike many X. campestris pathovars forms no pigment on sugar containing media.

The syndrome induced by Xanthomonas campestris pv manihotis on cassava plants is so complex that it appears unique among the known bacterial diseases of plants (Lozano & Sequeira, 1974b). Xcm penetrates the host through stomates and epidermal wounds (Lozano & Sequeira, 1974b; Lozano & Booth,1974). It invades and detroys the spongy mesophyll and enters the vascular tissues enabling the bacterial cells to move systemically through the plant in xylem vessels and possibly in phloem (Amaral, 1945). Vascular strands of infected petioles and stems become discoloured brown.The pathogen can cause extensive breakdown of parenchymatous tissues in leaves and young shoots.

Infection is more common in young plant tissues and usually symptoms develop 11-13 days after infection. In highly lignified old stems, the bacteria remain restricted to the vascular tissues, surviving for upto 30 months (Lozano, 1986).

The possibility that the pathogen spreads from one area to another and from one growing season to another by the use of infected stem cuttings has been suggested (Lozano & Sequiera, 1974b). Rain splashing is the most

important means of dissemination in localised areas. This accounts for the increase in disease severity in the rainy season reported by Drummond & Hipolito (1941). Some workers have suggested that the pathogen could be spread by movement of soil during cultural operations and by the use of contaminated tools (Drummond & Hipolito, 1941; Lozano, 1972).

Disease development slows during dry seasons as inoculum and conditions favoring penetration are lacking. The bacteria remain viable in affected plant tissues and previously exuded gum and become active again in the rainy season. The severity of the disease is enhanced by wide fluctuations in temperature but this phenomenon is not understood (Lozano, 1986).

5. The use of tissue culture to study host-pathogen interactions

Plant tissue culture techniques were originally developed for the study of fundamental problems of nutrition and morphogenesis, micropropagation, removal of virus and genetic engineering and have led to many important advances in these fields. More recently, as techniques and media have improved, it has become clear that tissue cultures provide simplified experimental systems for use in many other branches of plant science, including plant pathology (Ingram, 1977).

There are many potential advantages in using tissue culture techniques to study plant diseases, for the inciting organisms may be cultured together with their hosts in a controlled chemical and physical environment free from contamination. Media components can be defined or varied, temperature can be controlled and even gaseous components in vessels can be modified (Ingram, 1977). This offers the possibility of a simplified experimental system for investigating the structure and physiology of host-parasite interaction and, in the case of specialized parasites eg. some biotrophs, provides a means of maintaining continuous supplies of contaminant free propagules (Matthyse, 1983). In experiments where tissue cultures are used, large numbers of host cells may be exposed to a parasite without tissue injury, and host cell number and parasite inoculum density may be carefully controlled (Daub, 1986). Plant cells in culture are unwounded and free from extraneous organisms up to the moment of pathogen inoculation; therefore potential synergistic masking effects of other organisms can be ruled out. This is coupled with the possibility of rapid and homogenous application of inocula to all or most of the cells to be observed (Helgeson, 1983). The relative synchrony of the system assures that most cells (host or pathogen) are making the same response at the same time. In addition, metabolic inhibitors and precursors may be added to culture media and diffusible products of

interaction may be extracted with comparative ease. Such attributes are particularly important in studies of disease resistance and specificity, hormonal, enzymatic and other metabolic changes occurring after infection, the mode of action of pathotoxins and the maceration and killing of cells and tissues by secreted enzymes (Ingram, 1977).

Isolated protoplasts could be important when the presence of a cell wall may be a complicating factor, as in studies of infection and replication by viruses, the processes of host-parasite recognition and the effects of toxins on membranes and organelles (Daub, 1986).

The disadvantages of tissue culture systems include many of the same features cited as advantages. The very uniformity of the cell population which allows for timed sampling and uniform host cell responses also precludes the observation of the responses of differentiated cell types to the pathogen and of the interactions and communications among various plant cell types. Studies of acquired resistance in vitro would be impossible because of this lack of intercellular communication. Defensive reactions when studied in tissue cultures are dynamic reactions and static defensive barriers such as cuticles or prexisting inhibitors may not be present in tissue culture systems (Matthysse, 1983).

Physiological responses of the host, such as the

plugging of the vascular system associated with wilting or alterations in hormone transport, cannot be studied in tissue culture (Helgeson, 1983). Substances which in the infected plant would be present in localized areas with surrounding gradients of decreasing concentration, such as bacterial toxins or phytoalexins, will be uniformly distributed in a suspension culture (Ingram, 1977). They may be distributed differently in a callus culture growing on agar than in a plant organ. Even if there is no evidence for the involvement of a particular cell type in the response of a plant to a potential pathogen, it is necessary to determine that the tissue culture cells have not lost the ability to respond to the stimulus in the same way that cells in situ in the plant respond. Tissue culture cells may lose or change the ability to respond to exogenous auxins and cytokinins. Habituation, the loss of a hormone requirement for growth occurs with a frequency dependent on the species studied and the tissue culture medium used to maintain the cells. Some tissue culture lines have been reported to lose their ability to synthesise phytoalexins with increasing time in culture (Fett & Zacharius, 1982). Occurrence of genetic changes in culture (Scowcroft et al., 1983) is another disadvantage which can lead to new and abnormal gene expressions. A comparison of the relationship between fungal development and irreversible membrane damage (IMD) in lettuce cells penetrated by Bremia

lactucae in suspension cultures or in the epidermis of the cotyledon petiole and in the plant showed that there were differences between interactions occurring in vitro and and in planta. However, B. lactucae did produce characteristic infection structures in cultured cells and in cultivar/isolate interactions, the expected incompatibility or compatibility was clearly expressed (Fagg et al., 1991).

The balance of disadvantages and advantages could not be forecast in all cases: the ultimate value of a tissue culture system may lie in what it can reveal about events occurring in intact plants. Thus, frequent validations of findings in tissue cultures by looking for similar results with intact plants should be sought.

5a. Applications in the study of fungi and bacteria

5a(i). Obligate parasites

Plant tissue cultures have an advantage of studies of groups of fungi (eg.Plasmodiophorales, Chytridiales, downy and powdery mildews, smuts and rusts) which cannot be grown easily on artificial media. Dual cultures of obligate biotrophs and callus tissues of their hosts are potentially of value for the maintenance of supplies of aseptic inoculum, the cloning of isolates, the safe international transport of isolates, studies of host-parasite interactions and attempts to produce axenic

cultures (Ingram, 1976; 1977).

A number of downy mildew fungi have been cultured with their hosts: some of the examples are- Peronospora farinosa and sugar beet (Ingram & Joachim, 1971), P. tabacina and tobacco (Izard et al., 1964), Plasmopara viticola and vine (Morel, 1948) and Sclerospora sacchari and sugar cane (Chen et al., 1979). Dual culture of Plasmodiophora brassicae in naturally infected root tissue of cabbage has been reported by Williams et al. (1969) but it has been impossible to infect healthy brassica callus artificially with this organism (Dekhuijzen, 1975).

Attempts have been made without success to infect cells in culture with powdery mildew fungi in order to obtain an experimental system more amenable to manipulation than natural infections for the study of disease physiology (Webb & Gay, 1980).

5a(ii).Facultative parasites

Tissue culture systems could be used in studies of interactions between pathogens and their hosts at a cellular level. Growth of the tissue cultures and the type of system to use could vary with the particular study desired. Callus cultures, suspension cultures or plantlets could be used as appropriate.

Whilst there is always the possibility that plant cells in *in vitro* systems may not respond to pathogens or to their products in a manner that relates to responses *in planta*, there are many examples of them showing typical resistance and susceptible responses.

Work has been carried out to test the similarity of responses shown in tissue culture systems to the responses shown *in planta*. Investigations carried out by Nachmias *et al*. (1990) showed that bioassays using suspension cultured cells, sterile plantlets or root cultures of potato were comparable and reliable for the detection of *Verticillium* wilt tolerance. Roach & Garnett (1986) reported that cell suspensions of two cassava cultivars showed complete cell death when inoculated with two pathogenic strains of *Erwinia herbicola*. Callus cultures also have been used to demonstrate genotypic expression of resistance and susceptibility of plants. Helgeson *et al*. (1972; 1976) showed that tissue cultures of resistant tobacco cultivars inoculated with *Phytophthora parasitica* var *nicotianae* were colonized less rapidly and less extensively than tissue cultures of susceptible cultivars. Holliday & Klarmen (1979) also showed that resistant calli of soybean were colonized less than the susceptible by *Phytophthora megasperma* var *sojae*. Viseur *et al*. (1987) and Duron *et al*. (1987) used plantlets to determine the degree of fire blight resistance in pears and apples to *Erwinia amylovora* and

showed a clear distinction between the highly resistant and very susceptible clones.

The capacity of plant cells grown in tissue culture to produce phytoalexins has also been investigated from various plant species (Dixon, 1980). The phytoalexins include pisatin from Pisum sativum (Bailey, 1970), glyceollin from Glycine max (Ebel et al., 1976; Fett & Zacharius, 1982, 1983), phaseollin from Phaseolus vulgaris (Dixon & Fuller, 1976; 1978; Dixon & Bendall, 1978), medicarpin from Canavalia ensiformis (Gustine et al., 1978) and capsidiol from Nicotiana tabacum (Helgeson et al., 1978).

Hypersensitivity to incompatible pathogens has also been demonstrated with tissue culture systems. Atkinson et al. (1985) after inoculation of suspension cultured tobacco cells with an incompatible Pseudomonas sp. (P. syringae pv pisi) showed that suspension cultured tobacco cells provided a model system for studies of hypersensitivity. Also Matthyse (1987) inoculated suspension cultures of carrot and tobacco with an incompatible bacterium (P. syringae pv phaseolicola) and observed a hypesensitive reaction. Hypersensitivity was also elicited in potato suspension cultures and calli of resistant cultivars by P. solanacearum (Huang et al., 1989). Cultured cells were considered to show an HR like response if they showed a relatively fast cell death and

browning in cultures incubated with bacteria.

Tissue culture methods have also been used to understand crown-gall tumorigenesis caused by Agrobacterium tumefaciens (Butcher et al., 1980; Matthysse, 1983).

5b. Applications in the production of novel disease resistant plants

Tissue cultures form the basis of a number of techniques which have been developed to effect genetic changes in plants. It is feasible to induce and select for mutations among populations of cultured plant cells and then to regenerate plants from them (Brettell et al., 1980; Strauss et al., 1980; Grout & Weatherhead, 1980). This technique is likely to be particularly effective when haploid cells or protoplasts are used and where selection is for resistance to a pathotoxin. However, the nature of genetic changes involved should be determined.

The majority of agronomic crop plants are propagated by seed but an important minority (eg.potato, sweet potato, sugar cane) are vegetatively propagated. Some agronomic and horticultural plants either have no natural method of vegetative mutiplication (eg. oil palm) or can only be multiplied at very slow rates (eg. orchids). The use of tissue culture methods has made an

impressive contribution to the multiplication of such plants enabling the production of 'clonal crops' (Day, 1980).

In vitro selection for somaclonal varients insensitive to toxic metabolites produced by plant pathogens is a viable approach to obtaining disease resistant plants (Brettell et al., 1980; Daub, 1986; Hammerschlag, 1984) where toxins are a determinant of pathogenicity. Somaclonal variation describes the widespread phenomenon of varialtion of plants derived from any form of cell culture; generally associated with regeneration of plants from callus (Larkin & Scrowcroft, 1981). The feasibility of this approach depends on demonstrating that (i)a toxic metabolite is produced by the pathogen (ii)this metabolite is involved in disease development and (iii)the metabolite is active at cellular level. An additional prerequisite in in vitro selection is that of an efficient system for regenerating plants from callus and cell cultures (Hammerschlag, 1988).

It is important that the plants obtained from cultures selected for toxin resistance should be critically examined for expression and inheritance of the resistance character. Only in this way can it be determined whether the resistance observed is due to a mutational event or to epigenetic adaptation by the cultured cells to the presence of the toxin.

Culture filtrates of pathogens have been used as the selection pressure rather than purified toxins. Callus cultures of peach with Xanthomonas campestris pv pruni (Hammerschlag, 1988), cells from suspension cultures of alfalfa with Fusarium oxysporum and F. solani (Binarova et al., 1990), callus cultures of soybean for Phialophora gregata (Willmot et al., 1989) and callus cultures of elm for Ceratocystis ulmi (Pijut et al., 1990) are some of the examples where culture filtrates of the pathogen were used to select for resistance.

6. Pathogenicity determinants of bacteria

Aggressive assault upon the host, characteristic of fungi, is not apparent in bacterial invasion of plant tissue. Bacteria may be motile and capable of moving to the host in water, but the penetration of the host plant is accomplished either through natural openings eg. stomates, hydathodes, nectaries or through surface wounds or through breaks in fragile projections such as root hairs, trichomes and specialized exudative glands (Goodman, 1982).

Upon entry into the plant, extensive multiplication by bacteria occurs, either intercellularly or, as in some vascular diseases, in the xylem. Although bacteria have been shown to migrate in phloem by Lewis and Goodman (1965), and occasionally been detected in phloem cells,

they rarely multiply there (Huang et al., 1975). Both inter and intra cellular spread of the bacteria and the utilisation of host substrates is facilitated by various pathogenicity factors synthesised by the bacterium. A study of such factors that ensure successful pathogenesis of the host would help to give an insight to the mechanism of disease development.

Pathogenicity factors that allow colonisation of hosts by pathogens have been termed as 'basic compatibility' factors (Ellingboe, 1976). Little is known about the determinants of bacterial pathogenicity and specificity towards plants although factors such as cell envelope components, toxins, polysaccharides, depolymerases and auxins have been implicated.

6a. Toxins as determinants of pathogenicity

A toxin will be defined as a microbial product which directly damages or kills protoplasts of plant cells. This distinguishes toxins from agents which damage cells indirectly such as pectolytic enzymes and vessel occluding polysaccharides (Cooper, 1993). Although some effects such as membrane damage is direct, accumulation of ammonia could be the indirect toxic effect caused by inhibition of a chain reaction by the toxin. Toxins from plant pathogens are generally envisaged as low molecular weight because most known are less than 1000 Daltons; however, certain enzymes which are considered as toxins

and many of the compounds considered as toxins in animal pathology are larger than 50,000 Daltons (Stephen & Pietrowski, 1986).

6a(i).Nomenclature

Naming of toxins has been based on the organism which produces it, the susceptible host or the symptoms they cause.

Many plant pathologists have followed Rudolph's (1976) recommendations for giving trivial names for toxins. Thus, a name based on chemical structure, if known, is used for simple compounds such as fumaric or oxalic acids. However, many toxins have not yet been characterised; many are too complex for this system. Most trivial names of toxins therefore are based on the genus of the pathogen eg. cercosporin from Cercospora spp., fusicoccin from Fusicoccum amygdali or based on the species or pathotype if host adapted forms are known eg. syringomycin from many pathovars of Pseudomonas syringae and tabtoxin and phaseolotoxin from pathovars tabaci and phaseolicola. The toxin produced by Ceratocystis ulmi is named ceratoulmin based on both genus and species names.

Some host selective toxins are abbreviated to the first letters of the genus and species or pathotype eg. HV toxin from Helminthosporium victoriae and AK toxin from Alternaria alternata kikuchiana (Cooper, 1993).

6a(ii).Production of toxins

Production often occurs during a particular phase of growth, such as phaseolotoxin and tabtoxin in early log phase, A. alternata f. mali AM toxin in mid log phase, rhizobitoxin and syringomycin during stationary phase and lycomarasmin during autolysis. Production during early growth implies involvement early in infection; later production suggests virulence factor or an unimportant secondary metabolite (Cooper, 1993).

Production may be higher in static culture (syringomycin) or in shaken cultures (phaseolotoxin). Temperature optima for toxin production and the growth of the organisms might not always coincide (Cooper, 1993).Light and nutrients also can affect production of toxins.

6a(iii).Structure

Structures are now known for 11 host specific toxins (Knoche & Duvick, 1987; Daly, 1987), 5 bacterial toxins (Mitchell, 1981) and for over 200 other fungal toxins (Stoessl, 1981) of which only a few have been associated with disease. All characterised toxins are low molecular weight (< 1000 Daltons) except those causing wilt which have molecular weights between 10^4 and $2x10^6$ daltons (Strobel, 1977; Mitchell, 1981), but have very diverse structures. Bacterial toxins are usually amino acids or

oligopeptides; fungal toxins include phenolic, and heterocyclic compounds, terpenoids, glycosides,peptides, esters and linear polyketols (Cooper, 1993).

6a(iv).Biosynthesis

Knowledge of biosynthesis and regulation of toxins should help in maximising production in culture, and in evaluating regulation during infection by host nutrients or specific inducers. Inhibiting synthesis *in vitro* may reveal important alternative functions of the toxin. Suppresion of production *in vivo* may provide evidence of a role in pathogenesis. It is likely that synthesis will be regulated by processes similar to those that control production of other secondary metabolites, but little is known about them (Durbin, 1983).

6a(v).Role in pathogenesis

The ability of a pathogen to grow, reproduce and/or form overwintering structures in a host is controlled by its rate of utilisation of nutrients obtained from the host. To be successful the pathogen must break down and assimilate host materials and at the same time overcome resistance mechanisms the host might have. There are a number of potential functions that toxins could have, although there is little information as to which one(s) a particular toxin is assuming. Some of these include

that are high producers under one set of culture conditions may be low under another; other isolates of the same pathogen may follow the reverse pattern. b). simulation of the host environment is impossible and specific inducers/effectors of toxin may be provided by the host (Cooper, 1992) c).virulence may not always be correlated with the quantity of toxin produced in culture (Brian et al., 1952), even when the toxin is known to play a causal role in disease d). the level of toxin that accumulates in vitro under favourable conditions may be higher than the level required for disease development. If so, a correlation between quantity of toxin in vitro and virulence would not be expected.

A test of significance is to specifically eliminate the toxin from the biological system, leaving everything else the same, and observe what changes occur in disease initiation and development. Genetic manipulation either by mutational analysis or by naturally occurring variation, can be used for specific elimination of toxins from an experimental system (Yoder, 1980).

The above mentioned criteria cannot be considered as the ultimate factors in deciding the role of toxins in pathogenicity as there could be other aspects involved in the pathogenesis.

a).counteracting the initiation or maintenance of host resistance mechanisms b).damaging host cells which then release nutrients for pathogen growth c).providing a conducive micro environment for the pathogen (p^H, water potential) d).inhibiting secondary invasion by other microorganisms (Durbin, 1983).

Yoder, (1980) has suggested several criteria for evaluating the role of toxins in pathogenicity.

I.Host specificity

Toxins can be classified as host specific or host non specific (Patil, 1974). Host selective toxins have biological activity towards only one specific host whereas host non specific toxins can show toxicity towards all plants in general and in some cases to animals as well. For most of the host specific toxins, only an extreme specificity for susceptible plants would suggest a role in disease but even this specificity cannot be considered as valid since the high potency of these toxins makes them active against even disease resistant plants at higher but at still relatively low concentrations (Wood, 1976). A further difficulty is the apparent host specificity of materials that are not involved in disease. eg. the detergent digitonin and insecticide methomyl have similar specificity for mitochondria from T-cytoplasm corn as does HMT toxin (Yoder, 1980).

II. Presence in infected plant

Presence of the toxin in diseased tissues could mean only that a toxin is produced when a pathogen grows in the host; this does not necessarily indicate that the toxin is causing disease but rather than resulting from it. Similarly, toxins of host origin may result from infection. Inability to detect toxins in tissues may be due to lability, inactivation or binding by host tissue eg. HV toxin, fumaric acid (Cooper, 1993) or presence at concentrations below levels of detection.

III. Production at a key step in disease develpoment

Toxins should appear before or simultaneously with key steps in infection or symptom development. Some host selective toxins eg. from Helminthosporium victoriae and Alternaria alternata are released by germinating conidia in leaves before penetration and the first physiological changes detectable can be caused by these toxins alone (Cooper, 1993). This suggests that toxin action occurs during the infection process, but does not establish that infection cannot occur without toxin action.

In chronic diseases such as vascular wilts, toxins may be produced during late growth in plants or may even be products of autolysis but either of these may cause symptoms and thus function as virulence factors (Cooper, 1993).

IV. Induction of typical disease symptoms

Plants respond in a limited number of ways to disturbance and therefore a "typical" visible symptom may be caused by a factor that is involved in disease or by one that is not. eg. herbicide injury

Chlorosis can result from toxins with widely differing modes of action i.e. phaseolotoxin, tentoxin, rhizobitoxin, tagetitoxin as well as from factors such as water stress and ethylene. Wilting can result non specifically from low levels of many macromolecules, some of which are quite common in crude toxin preparations. Also, simulating a host-parasite interaction in vitro could be difficult as toxins could act with other mechanisms, may require a specific micro environment such as a particular ionic regime, and if cell bound, would need the parasite to deliver to vulnerable cellular sites. If possible, pure toxin should be used to avoid effects of contaminating compounds. There is also the problem of appropriate concentrations of toxin to apply and for how long (Cooper, 1993).

Ultrastructural changes caused by a toxin can be compared with those in infected tissue, but fixatives may penetrate more rapidly when treated with membrane damaging toxins. Electron microscopy could be used for observing/predicting cellular sites of action but the early changes have been only rarely been mimicked by the

toxin (Cooper, 1993). In H. maydis infected tissue, the first detectable effect is disruption of the tonoplast, whereas HMT toxin causes swelling of mitochondria. Also, the chloroplast damage in halo blight of bean is not reproduced by the toxin of P. syringae pv phaseolicola (Cooper, 1993).

Physiological symptoms may offer a better comparison of effects caused by the toxin and the pathogen. Some toxins known to be involved in disease also cause physiological changes found in diseased plants (Mitchell & Bieleski, 1977) whereas some toxic substances thought not to play important roles in disease do not cause physiological changes found in diseased plants (Kuo & Scheffer, 1964). But there are observations that do not fit this pattern. Stimulation of respiration by HMT toxin is not observed in diseased plants and PC toxin applied at low and then high doses kills seedlings without the electrolyte loss which characterises infection of sorghum by Periconia circinata (Yoder, 1980).

V. Correlation of virulence with quantity of toxin produced in vitro

Although this is often used, studies of toxin production in culture have limitations. These include a). influence of the medium composition and physical environment on toxin production (Comstock & Scheffer, 1972). Isolates

6a(vi).Genetic analysis to evaluate toxins as factors in pathogenesis

Genetic analysis can provide convincing evidence for toxins in disease. However, some pathogens and host plants do not lend themselves to genetic manipulation (Yoder, 1980).

Certain fungal pathogens have been analysed by recombination or mutation. For fungi such as Helminthosporium spp. and Perconia circinata pathogenicity and toxin production are closely related (Luke & Wheeler, 1955; Odvody et al., 1977; Scheffer & Pringle, 1961). Sexual crosses between Helminthosporium victoriae and H. carbonum and analysis of recombinants established roles in disease and specificity of their toxins (Scheffer et al., 1967).

Application of recombinant DNA techniques to certain bacterial pathogens has led to the identification and cloning of genes for production of toxins such as phaseolotoxin. Patil et al. (1974) and Staskawicz & Panopoulos (1979) obtained tox^- mutants of P. phaseolicola which indicated a role for phaseolotoxin in the virulence of the bacterium. Transposon mutagenesis has been used to create single site mutants which allows inactivation of specific genes. Tn5 induced tox^- mutants of P. syringae pv tomato indicated the toxin coronatine also to be a virulence factor (Bender et al., 1987). So

far only coronatine has been shown to be plasmid-encoded which precludes the manipulation of these extrachromosomal elements (Cooper, 1993).

6a(vii).A survey of toxins in disease

Toxins can be selective for pathogen susceptible cultivars of the host or host non-selective. None of the bacterial toxins have been reported to be host selective.

About 15 fungal toxins are known which are selectively toxic to pathogen susceptible cultivars, most of which are established as determinants of pathogenicity and specificity. Pathogen resistant cultivars are damaged but only at higher concentrations. Most host selective toxins known are from species of Helminthosporium and Alternaria (Yoder, 1980).

6a(vii)1.Bacterial toxins

All the bacterial toxins known are host non selective and all that have been clearly linked with pathogenesis come from Pseudomonas syringae pathovars except rhizobitoxin from the N_2 fixing Rhizobium japonicum (Cooper, 1993).

P.syringae pv tabaci

The toxin produced by P. tabaci has been traditionally referred to as the "wildfire" toxin (Patil,

1974). Two other pathovars of Pseudomonas ie. P. syringae pv coronafaciens a pathogen on oats and other cereals and another pathogenic to timothy also produced tabtoxin (Patil, 1974; Sindon & Durbin, 1970).

Tabtoxin appears to be the sole cause of chlorosis but not of pathogenicity because tox(-) mutants of pv tabaci colonise tobacco leaves but produce no chlorosis or any of the characteristic physiological effects found in chlorotic haloes (Turner & Taha, 1984). Tabtoxin is a peptide protoxin which is hydrolysed by plant or bacterial enzymes to release the chlorosis inducing component, tabtoxinine B lactam (Uchytil & Durbin, 1980). This inactivates glutamine synthetase (Thomas et al., 1983). Toxin action also has been reported to cause light dependent disruption of chloroplast structure. Chlorophyll content decreases by 35% within 2 days (Sinden & Durbin, 1968). These effects have been hypothesised to result from a build up of ammonia to toxic levels (Thomas et al., 1983). Ammonia accumulation has been observed by Turner (1981) to be the direct consequence of glutamine synthetase inhibition . Ammonia (as NH_4) reaches millimolar levels in infected tissue and is related to chlorosis and necrosis. It disrupts chloroplast thylakoid membranes and may act by uncoupling photophosphorylation or diverting protein into ammonia N (Durbin, 1983; Gilchrist, 1983).

P. syringae pv phaseolicola

P. phaseolicola the causal agent of halo blight of beans (Phaseolus vulgaris), also produces a peptide toxin, phaseolotoxin, which is responsible for production of the chlorotic haloes in infected bean tissues (Patil et al., 1972). Other legumes affected include pigeon pea (Hayward et al., 1983), mung bean and Dolichos spp. (Mitchell, 1978).

Phaseolotoxin was identified as (N^6 phosphosulphamyl) ornithylalanyl homoarginine (Mitchell, 1984) and specifically inhibits ornithine carbomyltransferase (OCTase) from a variety of organisms. OCTase converts ornithine and carbomyl phosphate to citrulline in the urea cycle. The sulphamyl phosphate moiety binds to the enzyme and thus competetively inhibits binding of carbomyl phosphate (Gilchrist, 1983).

Naturally occurring or induced $tox^{(-)}$ mutants are observed to mutiply in leaves and produce typical water soaked lesions but do not cause chlorosis or spread systemically in the plant (Panopoulos & Staskawicz, 1981). Chlorosis may result from a decrease in protein synthesis due to arginine deficiency, or from de-repression in pyrimidine synthesis owing to citrulline deficiency (Mitchell, 1984).

Pseudomonas syringae - various pathovars

Coronatine- Coronatine is a terpenoid toxin produced and has been detected in Italian ryegrass infected with pv atropurpurea (Nishiyama et al., 1977), soybean leaves infected with pv glycinea (Gnanamanickam et al., 1982) and in tomato leaves infected with pv tomato (Mitchell, 1984). Application of the toxin to leaves caused chlorosis and browning characteristic of infection. This and the production by many pseudomonads suggest a role in virulence. Bender et al. (1987) found that Tn5 mutants of pv tomato produced the same number of lesions in tomato leaves as did the wild type but these lesions were ca. 1/3 the size of comparable wild type lesions; also the latter continued to expand for a longer time.

Tagetitoxin- P. syringae pv tagetis causes necrotic leaf spots sometimes with chlorotic haloes as well as an apical chlorosis in several species within the family Compositae; African marigold, French marigold, sunflower, artichoke dandelion and compass plant (Rhodehamel & Durbin, 1989). The bacterium produces a toxin which has a cyclic hemithioketal structure named tagetitoxin which is responsible for both types of chlorosis (Durbin & Mathews, 1987). The toxin acts by inhibiting chloroplast RNA polymerase (Lukens et al., 1987).

Syringomycin- Pathogenic strains of P. syringae pv syringae produce one of two low molecular weight peptide toxins. Syringomycin is produced by isolates from a wide

variety of hosts from more than 30 genera, whereas citrus isolates produce syringotoxin which is chemically distinct from syringomycin but has similar phytotoxicity and biological properties (Gross & Cody, 1985).

Rhizobium japonicum

Certain strains produce a toxin in culture, and in root nodules and it cause chlorosis in developing leaves where the toxin had been detected (Owens & Wright, 1965). It is 2 amino-4(2 amino-3-hydroxy-propoxy)-trans-but-3-enoic acid (Mitchell, 1981) and causes chlorosis. Rhizobitoxin blocks β cystathionase, the enzyme in the methionine pathway that converts cystathione to homocysteine (Giovanelli et al.,1972).

Toxins produced by Xanthomonas

X. campestris pv malvacearum - This bacterium causes bacterial blight of cotton but little is known about the basic mechanisms involved in disease production (Hopper et al., 1975). Lakshmanan & Krishnamurthy (1979) indicated that a non enzymatic factor produced by X. campestris pv malvacearum in culture filtrates may be involved in pathogenesis. Work by Krishnamurthy (1976) with culture filtrates of X. malvacearum on symptom production and respiration of cotton suggested the presence of a toxic principle. A dialysable, thermostable, aromatic compound has been isolated and

semipurified from culture filtrates of the bacterium and from the necrotic areas of infected cotton leaves (Lakshmanan & Kathirvel Pandian, 1988).

X. campestris pv manihotis

Perreaux et al. (1982) suggested an involvement of toxic substances diffusing from the angular spots in diseased lamina tissues because of the rapidity of the extension of diseased areas and also because they could not isolate bacteria from them. Blight symptoms similar to those induced by the bacteria have been reproduced with extracts from a broth culture of X. manihotis (Perreaux , 1979). The toxin was identified as 3 methyl thiopropionic acid (MTPA) by mass spectrometry, infra red spectroscopy and C,H,N analysis. This was confirmed by chemical synthesis. MTPA was also detected in extracts of cassava leaves naturally or artificially infected by X. manihotis (Perreaux et al., 1986) using GLC. The highest toxin concentration was associated with the appearance and extension of the blight symptoms in artificially infected leaves. MTPA was also detected in necrotic tissues.

Ewbank and Maraite (1990) showed that incubation of the bacterium with either C^{14} methionine or 3,4 ^{14}C KMBA (2 keto 4 methyl thio butyric acid) led to the production of ^{14}C MTPA and ^{14}C MTAA (3 methyl thio acrylic acid).

They deduced that methionine is catabolised by X. campestris into MTPA via transamination and subsequent decarboxylation of the intermediate keto acid KMBA.

6b. Cell envelope components

The structure of the cell envelope of bacteria of the genus Xanthomonas appears to be similar to that of the Gram-negative cells as reviewed by Costerton et al., (1974). The cell envelope can be divided into the plasma membrane, the peptidoglycan layer and the outer cell membrane. The protoplast is bounded by the plasma membrane which is composed of 20-30% phospholipid and proteins. About a fifth of the total cell proteins are located in the plasma membrane, which plays an important role in cell metabolism.

The plasma membrane of Gram-negative bacteria is enclosed by a rigid peptidoglycan layer which serves to counteract the turgor pressure of the protoplasm, preventing osmotic lysis in a hypotonic environment. Bound to the peptidoglycan layer by lipoprotein is the outer cell membrane which is composed of phospholipid, lipopolysaccharides (LPS) and proteins. Proteins in this layer are very different to those found in the inner membrane.

The periplasmic space is the aqueous compartment between the plasma membrane and the peptidoglycan layer.

A number of enzymes are located within the periplasmic space or in the outer cell wall membrane (Ramaley, 1979). Since periplasmic enzymes are external to the inner membrane, they could be considered as extracellular enzymes. Enzyme secretory mutants are often unable to export the enzymes from the periplasm to the external environment (Andro et al., 1984; Dow et al., 1987).

Only relatively few periplasmic enzymes perform a degradative function. These include a polygalacturonic acid trans-eliminase located in the periplasmic space of Erwinia rubrifaciens (Gardner & Kado, 1976) and a number of degradative enzymes in E. coli (Heppel, 1967; Nossal & Heppel, 1966).

6c. Lipopolysaccharides (LPS)

LPS are major components of the outer cell membrane of gram-negative bacteria and are thought to be located in the outside of the outer membrane bilayer, in which they are anchored by hydrophobic interactions of the lipid A moiety of the molecule with membrane phospholipid and protein (Muhlradt & Golecki, 1975).

LPS are complex molecules, with molecular weights of more than 10,000 daltons. They vary widely in chemical composition both within and between groups of gram-negative organisms. Each LPS molecule is composed of three distinct regions; lipid A, the R core

polysaccharide and the O-antigen side chain polysaccharide.

LPS serves as the receptor for the adsorption of many bacteriophages and bacteriocins. Phages have been identified which recognise specific sites either in the core (Lindberg, 1977) or in the O antigen side chain region (Israel et al., 1972; Lindberg et al., 1978) of the LPS molecule. In these cases, changes in LPS structure at the specific site of binding confer phage resistance upon the mutant strain. A bacteriophage-resistant mutant of Erwinia amylovora was found to lack O-antigenic sidechains on the LPS (Ray et al., 1986).

LPS plays a dominant role in the functioning of the outer membrane (Inouye, 1979) and is also important in maintaining the structural stability of the outer membrane. LPS defective or rough strains of bacteria are known to leak periplasmic enzymes into the culture medium (Chatterjee et al., 1976; Lindsey et al., 1973).

LPS has been suggested to have an important role in bacterial-plant associations for Agrobacterium tumefaciens (Matthysse et al., 1978), Pseudomonas solanacearum (Chatterjee & Vidaver, 1986) and Erwinia stewartii (Huang, 1980). The lipid A-R core segment of LPS was reported to induce resistance in tobacco to P. solanacearum. There is however evidence, reviewed by Panopoulos & Peet (1985) that LPS is not the inducer of

the HR interaction. It does however appear to serve as an attachment molecule to the plant cell surface and therefore, may be indirectly involved in the elicitation of the HR.

6d. Extracellular polysaccharides (EPS)

Production of polysaccharides outside the cell wall is common in many genera of bacteria. These exracellular polysaccharides (EPS) can form an organised capsule around the cell or can be shed into the environment as slime (Coplin & Cook, 1990). Although synthesis of EPS may be optional depending on growth conditions, it is frequently a major determinant in the ability of a bacterium to colonize a given niche (Costerton et al., 1987). Two properties of EPS help to determine their functions (Ferris & Beveridge, 1985). First, capsular polysaccharides are highly hydrated. this protects the bacterium against desiccation and prevents hydrophobic molecules from penetrating the capsule and arriving at the cell membrane. For example, encapsulated bacteria are more resistant to the action of detergents and hydrophobic polypeptide antibiotics. Second, the acidic EPSs produced by most plant pathogenic bacteria are highly anionic. As such, they can act as ion exchange resins, concentrating minerals and nutrients in the vicinity of the cell or binding toxic elements (Norberg & Persson, 1984). Another important function of capsular

polysaccharides, in soil and aquatic environments, is to help bacteria adhere to inert or biological surfaces (Norkrans, 1980; Ramphal & Pier, 1985; Marcus & Baker, 1985), where higher concentrations of nutrients may be found.

Plant pathogenic bacteria produce a number types of EPS which can be placed under two major classes: homopolysaccharides of which the simplest is levan found in various Pseudomonas spp. (Fett et al., 1989) and E. amylovora (Bennnett & Billing, 1980). It is synthesised extracellularly from sucrose by the enzyme levansucrase (Gross & Rudolph, 1987). Other simple polymers are glucans produced by some gram-positive bacteria (Stoddard, 1984) and alginate produced by pseudomonads (Fett et al., 1989; Ohman, 1986). Heteropolysaccharides such as xanthan produced by Xanthomonas spp. are more complex. These usually consist of repeating units of 2-4 monosaccharides (Chatterjee & Vidaver, 1986). Both homo and heteropolysaccharides can possess long side chains.

The studies on xanthan gum (a commercially available product) of X. campestris pv campestris provide the most complete model for EPS synthesis because it is the only system in which both biosynthetic genes and their enzyme products are known (Coplin & Cook, 1990). The repeating unit of xanthan is a pentasaccharide composed of 2 glucose, one glucuronic acid and 2 mannose moieties. The

glucose forms a β-1-4 linked backbone, and a Man-GlcA-Man side chain extends from alternate Glu moieties. The Man residues in the side chain are acetylated and pyruvylated to a specific alternating pattern.

6d(i).Genetics of EPS synthesis

Large clusters of EPS biosynthetic genes have been reported in *E. coli* (Trisler & Gottesman, 1984), *X. campestris* (Barrëre *et al*., 1986; Harding *et al*., 1987; Hotte *et al*., 1990; Thorne *et al*., 1987; Vanderslice *et al*., 1989), *Rhizobium* spp. (Chen *et al*., 1988) and *E. stewartii* (Coplin & Majerczak, 1990; Dolph *et al*., 1988). In each case DNA that complemented EPS mutants was cloned and mapped by subcloning and transposon mutagenesis; regulatory mutants were identified by their phenotype or effect on reporter gene fusions. Molecular cloning techniques have been used in *P. solanacearum* to demonstrate that EPS was necessary for virulence (Staskawicz *et al*., 1983). EPS deficient mutants of *E. stewartii*, the vascular wilt pathogen of corn, were unable to cause systemic wilting symptoms and hence EPS appears to be a pathogenicity factor in this disease too. Work by Daniels *et al*. (1989) suggested that at least two positive regulators and a negative regulator control xanthan gum synthesis in a wild type strain of *X. campestris pv. campestris*. Daniels *et al*. (1989) also isolated nonpathogenic mutantsof *Xcc* and found one of

them to be defective in the production of extracellular protease and pectate lyase and severely depressed in the synthesis of EPS, glucanase and amylase. Genetic analysis of the corresponding locus revealed a cluster of five positive regulatory genes that have co-ordinate effects on all four enzymes, EPS and pathogenicity.

6d(ii). The role of EPS during pathogenesis

Many plant pathogenic bacteria need to grow and survive in soil, in irrigation water or on plant leaves or roots to complete their disease cycle. Protection against a hostile environment could be one reason for plant pathogens to produce EPS; however, it could be an important factor in pathogenicity and virulence. EPS has been shown to play a vital role in attachment and infection by *Agrobacterium tumefaciens* (Kamoun *et al.*, 1989) and *Rhizobium* spp. (Chen *et al.*, 1988; Djordjevic *et al.*, 1987; Leigh *et al.*, 1985). Capsules and slime may act as barriers to host defences, such as phytoalexins and block recognition events (Bradshaw-Rouse *et al.*, 1981; Romeiro *et al.*, 1981). In leaf spots, bacterial slime formed in the intercellular spaces may hold the water and nutrients released from damaged cells and thereby contribute to both watersoaking symptoms and the creation of a favourable environment for bacterial multiplication (Coplin & Majerczak, 1990).

It is assumed that the presence of EPS in the intercellular spaces of leaf mesophyll causes a gel formation, alone or in association with plant substances. This gel may inhibit or weaken the resistance reaction of the plant due to several effects:

I).Prevention of contact between bacteria and plant cell walls. Morphological contact between host and parasite is often necessary to induce resistance reactions.

II).Inhibition of bacterial agglutination Agglutination activity has been reported to correlate with the degree of resistance (El-Banoby & Rudolph, 1980; 1981). Higher agglutination caused less virulence of bacteria and therefore prevention of agglutination by EPS may be one mechanism to induce compatibility (Rudolph et al., 1989).

III).Prevention of desiccation Persistent watersoaked spots arise from the attraction of water by EPS in plant leaves. Since bacteria need water for colonisation of the normally air filled intercellular spaces, the generation of a watery environment may be important (El-Banoby & Rudolph, 1979).

IV).Protection against bacteriostatic compounds Most of the bacteriostatic substances such as phytoalexins are lipophilic (Rudolph et al., 1989). Therefore it can be assumed that these compounds are not taken up by bacterial cells which are separated from plant cells by

water-rich 'spacer' gels.

V).Decrease of oxygen tension The replacement of air in the intercellular spaces of the leaf mesophyll by bacterial EPS should result in a nearly complete blocking of the gas exchange of the host cells (Rudolph et al., 1989).

VI).Occlusion of vessels Whether wilting is a function of EPS size and viscosity has not been clarified since very little is known about how the structure of EPS influences pathogenicity (Coplin & Cook, 1990).

EPS from phytopathogenic xanthomonads and pseudomonads has been demonstrated to cause persistence of watersoaking in leaves of susceptible host but not induction of water soaking as the ambiguous title of El-Banoby & Rudolph's (1979) paper implies. Persistence of watersoaking only occurred in leaves of a compatible host plant and was later claimed to be host specific at cultivar level (El-Banoby & Rudolph, 1979; El-Banoby et al., 1980). Ultrastructural examination of the response of susceptible and resistant bean cultivars to EPS of P. phaseolicola suggested that enzymic degradation of bacterial EPS occurred only in resistant leaves, and this effect could be reproduced using intercellular fluids in vitro (El-Banoby et al., 1981). The authors suggested that the persistence of watersoaking in susceptible tissues occurred as a result of the trapping of water

from the transpiration stream rather than a direct increase in host cell permeability.

The production of EPS has been implicated in pathogenicity of Erwinia amylovora but Bennett (1980) and Billing (1984) demonstrated that it is not the sole determinant of pathogenicity. Billing (1984) postulated that EPS may function to maintain a supply of free water for bacterial growth and therefore was necessary in addition to a virulence factor that causes ion leakage from cells. EPS in this plant pathogen would therefore constitute in Ellingboe's terminology (1976) a 'basic compatibility' factor.

Wakimoto (1985) also implicated Xanthomonas EPS (xanthan) as a pathogenicity factor in rice-X. campestris pv oryzae interaction. Phage-infected strains which produced a greater amount of EPS than uninfected strains, incited symptoms on rice leaves earlier and more severely than uninfected strains. Xanthan secretion was the only detected change caused by phage-infection, but it is probable that the latter has pleiotrophic effects.

Conversely, Barrëre et al. (1986) suggested that functional EPS genes did not appear essential in pathogenesis of X. campestris pv campestris because in vitro EPS$^-$ mutants did incite black rot on turnip seedlings. EPS genes were cloned but their function has

remained uncertain as it was not shown that the mutants were unable to produce EPS *in planta*.

6e. Extracellular enzymes

Pathogen produced cell-wall degrading enzymes have been implicated in numerous plant diseases (Cooper, 1984). Most of the CWDE reported in bacterial-plant interactions are concerned with pectolytic enzymes (Basham & Bateman,1975; Zucker *et al.*, 1972; Stephens & Wood, 1975; Collmer *et al.*, 1985; Kotoujansky *et al.*, 1985) and to a lesser extent proteolytic enzymes (Friedman, 1962; Keen *et al.*, 1967; Bashan *et al.*, 1986) and cellulolytic enzymes (Kelman & Cowlings, 1965; Kotoujansky *et al.*, 1985).

6e(i). Pectolytic enzymes

In primary walls of dicotyledonous plants, rhamnogalacturonan functions as the key matrix polymer. This is composed of linear polymeric chains of ~ 1,4 linked galacturonic acid interspersed with 1,2-linked rhamnose. The carboxyl groups of galacturonic acid could be methylated or cross linked by Ca^{2+}, which can result in gel formation and thereby affect wall properties and amenability to CWDE (Cooper, 1987). Side chains of the neutral polymers arabinan and galactan can be linked to this backbone.

Rhamnogalacturonan is degraded by hydrolytic

polygalacturonases (PG) or by lyases (PL) which result in an unsaturated bond between carbons 4 and 5 at the non-reducing end of the broken chain. Both may attack the chain at random (endo-) or terminally (exo-) but some combine the two modes of action and attack chains at random, then release mono or oligomers (multiple attack) (Cooper & Wood, 1975). Specificity is shown for regions either with methylated or free carboxyls. PGs usually have acidic pH optima (4-5) whereas lyases are most active at high pH (8-10) and have a requirement for Ca^{2+} unlike the PGs. The methoxy groups on pectinic acids are removed by pectin methylesterase (PME) which can therefore facilitate the action of depolymerases specific for free carboxyls.

Highly purified endo but not exo-acting pectic enzymes are able to macerate and kill parenchymatous plant tissues (Basham & Bateman, 1975a; Stephens & Wood, 1975; Basham & Bateman, 1975b; Liao & Wells, 1987; Liao, 1989;). The mechanism of cell killing is generally considered to be that the enzyme weakened wall can no longer counteract the pressure exerted by the protoplast resulting in a damaged plasmalemma (Cooper, 1983). However, this may be an oversimplication as ion leakage indicative of cell injury occurs almost immediately when tissues are exposed to pectic enzymes (Hislop *et al.*,1979; Basham & Bateman, 1975a; Stephens & Wood, 1975; Basham & Bateman, 1975b; Ikotun, 1987). An additional

effect of pectic enzymes on plants is their ability possibly through generation of pectic oligosaccharides to stimulate defence reactions (Gardner & Kado, 1976).

Of the various pectolytic enzymes produced by phytopathogenic bacteria, the pectate lyases of Erwinia chrysanthemi and E. carotovora subsp. carotovora have been studied most intensively and an essential role for these enzymes in pathogenicity is suggested (Collmer et al., 1985; Kotoujansky et al., 1985). Although there has been rapid progress in understanding the regulation of Erwinia pectic enzymes in culture, little is known about their regulation in the plant.

The main regulatory controls on polysaccharidase synthesis are induction, effected specifically by the monomer, or in some cases the dimer, of the polymeric substrate, or catabolite repression caused non-specifically by a wide range of energy sources (Collmer et al., 1982; Cooper & Wood, 1975; Cooper, 1977). Low basal synthesis by releasing specific inducers allows 'recognition' of wall polymers encountered by pathogens (Cooper, 1987).

Extracellular pectate lyases of Erwinia spp. can be resolved by isoelectric focussing into two or more isozymes depending on the strain. The conferral of the pectate lyase phenotype by multiple isozymes originally

presented a problem to genetic analysis of the genes involved, but was overcome by Collmer et al. (1985) using gene manipulation after molecular cloning of pel genes. Kotoujansky et al. (1985) used LacZ fusins to study the expression and regulation of each E. chrysathemi pel gene and mutants were created that were deficient in just one of the five isozymes. Two alkaline isozymes pel E and pel D were found to be essential for pathogenicity; pel D$^-$ and pel E$^-$ mutants were able to kill the inoculated Saintpaulia ionatha (African violet) leaf, but the bacterium did not spread to the rest of the plant. The remaining enzymes, pel A, pel B and pel C were not necessary for pathognensis, but did increase symptoms. The pel B and pel C enzymes are both neutral isozymes and have a high level of homology with each other and also with an E. carotovora pel gene, suggesting that these isozymes may have arisen from a common ancestral pel gene. The pel D and pel E genes are more 'endo' acting than the other three isozymes and these genes were found located in a gene cluster together with pel A and a pectinmethylesterase gene (Kotoujansky, 1987).

Pectate lyase secretory mutants of E. chrysanthemi were also found to be cellulase-deficient suggesting that these enzymes are under co-ordinate synthesis or secretion. A similar finding was reported for Xanthomonas campestris pv campestris (Daniels et al., 1984; Dow et al., 1987); mutant 8237 was deficient in the secretion of

pectate lyase, protease and carboxymethyl cellulase. This gives some hope for obtaining enzyme-deficient mutants in spite of the existence of more than one structural gene.

Many factors concerning pectic enzyme production and secretion such as why some E. chrysanthemi strains possess so many isozymes and why some bacteria produce pectic enzymes at levels comparable to the above Erwinia spp. but are not plant pathogens are still not understood. These saprophytes may lack other basic compatibility factors, or the ability of Erwinias to invade plants may be due to the rate of pectate lyase production at infection sites or the adaptation of those enzymes to particular forms of galacturonan within cell walls (Collmer et al., 1982).

Little is known about pectic degedation by Xanthomonas spp. in comparison with the knowledge available for some other bacterial and fungal genera. The nature of diseases caused by xanthmonads generally does not suggest pectolytic enzymes as important pathogenicity factors; cell wall dissolution symptoms such as tissue maceration are not typical. However, several reports attest to the pectolytic activity of the xanthomonads (Burkholder & Starr, 1948; Sabet & Dowson, 1951; Smith, 1958; Dye, 1960; Abo El Dahab, 1964; Nasuno & Starr, 1967; Starr & Nasuno, 1967; Ikotun,1984a; 1984b; Liao & Wells, 1987; Dow et al., 1987; 1989; Angeles-Ramos et

al., 1991; Beaulieu et al., 1991).

Dye (1960) demonstrated that pectolytic activity was present in some of the 145 cultures tested (representing 38 Xanthomonas spp.) but was however, variable among strains. Nasuno & Starr (1967) purified polygalacturonic acid transeliminase from the culture fluids of X. campestris. Starr & Nasuno (1967) reported that 10 out of 19 Xanthomonas cultures tested liquefied a nutrient pectate gel and 7 of these formed pectate transeliminase (some inducibly on pectin and a few constitutively on glucose). 17 of the spp. showed a detectable pectinesterase activity but none formed the hydrolytic polygalacturonase or pectate transeliminase. According to their report X. manihotis showed a good growth on pectate gel medium and brought about prompt liquefaction of the gel.

Abo El-Dahab (1964) found inducible pectin methylesterase activity in X. malvacearum culture filtrates and Ikotun (1984a) also obtained similar production by X. manihotis (Xcm). X. cassavae, is also reported to produce pectin methylesterase, pectate lyase and small amounts of polygalacturonase (Ikotun, 1984b); a possible role for these enzymes in pathogenesis was suggested as they were detected in infected host tissues. Beaulieu et al. (1991) reported that 22% of 522 strains of X. vesicatoria tested were pectolytic on a sodium

polypectate medium and the pectolytic activity was attributed to the secretion of a single pectate lyase with a pI of 8.8.

Some post-harvest rots of plant crops have been attributed to pectolytic xanthomonads (Liao & Wells, 1987). Five strains of bacteria with properties conforming to those of X. campestris were found to secrete pectate lyase, pectin esterase and polygalacturonase in medium containing pectin or polygalacturonic acid but not in a medium containing glucose indicating induced production.

6e(ii).Proteases and phospholipases

Proteins occur in plasma membranes, primary cell walls and middle lamellae and phospholipids in membranes of higher plants (Ginzburg, 1961; Lamport, 1965) and thus their degradation could be involved in pathogenesis.

Although disruption of isolated protoplasts by proteases and phospholipases has been reported in some interactions, disruption of 'protoplasts' in intact tissue has not been obtained with either proteases or phospholipases and thus these enzymes may only work subsequent to CWDE such as pectinases (Bateman & Basham, 1976). To date, insuffient information exists on these enzymes to conclude as to their role in pathogenesis.

Many bacteria such as spp. of Xanthomonas and Pseudomonas are capable of hydrolysing proteins in culture but few workers have implicated proteolytic activity in bacterial pathogenicity. Gainor & Crisley (1961) did however, report increased protease activities in tobacco crown gall tissues induced by Agrobacterium tumefaciens. Friedman (1962) postulated that increased virulence of Erwinia carotovora could be attributed to increased proteolytic activity and Keen et al. (1967) reported that Pseudomonas lachrymans, the causal agent of cucumber angular leaf spot, produced a caseinolytic protease during pathogenesis and and that there was some correlation between pathogenicity and protease production in culture.

Proteolytic activity has also been reported for infections by Xanthomonas spp. Reddy et al. (1971) found that protease activity was 20 times greater in extracts from susceptible plants inoculated with X. alfalfae than in extracts from non-inoculated control plants. In addition they found that although broths based on crude extracts from resistant and susceptible alfalfa plants supported nearly equal bacterial growth, protease activity was greater in broth from the susceptible plants. More recently a X. campestris pv campestris non-pathogenic mutant was found to be defective in secretion of protease, pectate lyase and carboxymethyl cellulase (Daniels et al., 1984). Enzyme activities and

pathogenicity on turnip seedlings were restored by a wild type cosmid clone. It was suggested that these enzymes may be important in pathogenicity. However, it is possible that there were other undetected differences from the wild type and the mutant which contributed to the loss of pathogenicity. Dow et al. (1990) fractionated two proteases from culture supernatants of wild type X. campestris pv campestris and showed that a protease deficient mutant which lacked both these proteases to be less virulent in pathogenicity tests on mature turnip leaves. This suggests that these two proteases have a role in black rot pathogenesis.

6f. Siderophores

Siderophores are defined as low molecular weight, ferric specific ligands designed for the solubilisation and transport of iron (III) in microbial species (Neilands, 1982; 1984). Siderophores complex ferric iron, and are generally either hydroxamates or phenolates (Neilands, 1982). The presence of phenolate siderophores has been demonstrated in Aerobactor aerogenes, E. coli and certain species of Salmonella (O'Brien & Gibson, 1970; Pollack & Nielands, 1970).

6g. Growth regulators as pathogenicity determinants of disease

There are various classes of compounds with growth regulating properties that occur naturally in plants. Some examples are auxins, cytokinins, ethylene and Abscisic acid (Pegg, 1981b). A number of hormone-like molecules are known as pathogenic toxins, viz. helminthosporol from Drechslera sorokiniana (Briggs, 1966; Hashimoto & Tamura, 1967) fusicoccin from Fusicoccum amygdali (Ballio et al., 1964), hadicin from Helminthosporium frequentans, the diaporthins and the ophiobolins.

The best evidence for a positive and exclusive role for an auxin eg. indoleacetic acid-IAA in pathogenesis is reported by Smidt and Kosuge (1978) on oleander galls induced by Pseudomonas savastanoi. Beltra (1961) reported that galls on olive contained increased levels of a growth-promoting substance.

The pathological systems most studied that are associated with cytokinins are fasciation and leafy galls typified by Corynebacterium fascians (Thimann & Sachs, 1966; Sachs & Thimann, 1967), crown gall caused by Agrobacterium tumefaciens, club root caused by Plasmodiophora brassicae and maize smut caused by Ustilago maydis (Pegg, 1981).

Zeigler et al. (1980) reported of the involvement of a specific gibberellin in cassava superelongation disease caused by Sphaceloma manihoticola. When 30ug authentic gibberellic acid was applied to young cassava plants, symptoms identical to natural infection were obtained.

Phytohormones have not been reported to be involved in diseases caused by Xanthomonas spp.

The following study was carried out with a view of critically investigating the mechanisms involved in the host-pathogen interaction in an attempt to understand the pathogenicity factors involved in successful pathogenesis of cassava by Xcm.

MATERIALS AND METHODS

1. Plant material, growth and maintenance

Plants of cultivars MCol 113 and MCol 22 were propagated from clonal stocks and MVen 77 and MNGA 1 from stakes supplied by CIAT, Colombia. The growth habit of most cutivars was similar except that MCol 22 was more vigourous.

The four cassava cultivars used in this study have already been rated for their response to inoculation with strain 1060 0f Xcm. i.e.-
MVen 77 - resistant, MNGA 1- resistant (CIAT)
MCol 113- susceptible, MCol 22- susceptible
(Lozano and Laberry, 1982).

Propagation was from stem stakes which were cut into 15-20cm lengths,(each possessing up to 5-10 nodes) and their lower ends were dipped in a commercial rooting powder containing IBA (Seradex, May and Baker Ltd.). Stem cuttings were planted in a mixture of peat and perlite (3:1 v/v) contained in 12.5 cm diam plastic pots. These were maintained initially in a propagator with high humidity and temperature(80% and 28-30°C). When shoots and roots were initiated these cuttings were transferred to 20cm diam pots containing John Innes No. 2 compost and a slow release fertiliser (Vitafeed 101). Plants were grown under glass-house conditions with a photoperiod of

12 hours with natural lighting supplemented by mercury lamps from October to March. Temperature varied between 25-30°C.

2.Tissue culture of cassava

a).Media preparation

Murashige and Skoog (MS) medium (1962) (Flow laboratories) was used without hormones (basal medium) or hormone stock solutions were added to obtain the desired concentration. Heat stable plant growth regulators and compounds were added before the medium was autoclaved, whereas heat labile compounds were filter-sterilized through a Sartorius membrane filter of pore size 0.2 um and added later. Sucrose (Analar grade) at 2% w/v and agar (Oxoid) at 0.6% w/v were added unless otherwise stated. The media were made upto volume and p^H adjusted to 5.7 with 1M NaOH or 1M HCl.

The media were dispensed to screwtop bottles (100 ml, 250 ml, 500 ml or 1000 ml as appropriate) and sterilised by autoclaving for 15 miniutes at 121°C and 1.4 Bars pressure. After cooling, the media were poured into Petri-dishes (25 ml to a 9 cm diameter dish or 15 ml to a 5 cm dish), Sterilin jars (25 ml/100 ml jar) or sterilin Petri squares (3 ml/division) as required. The poured plates and jars were stored in the dark at room temperature until required.

b). Sterilisation of plant material

The explant and adjacent tissues were removed from glasshouse grown plants. Sterilisation and all subsequent *in vitro* procedures were carried out in the sterile environment provided by a laminar flow cabinet. (Microflow Pathfinder Ltd.).

Table 1 shows details of the type, sterilisation and source of explants used in the study. Tissues were surface sterilised by sodium hypochlorite solution (5% solution, 0.1% available Cl_2) with 1-2 drops of Tween 80 (BDH). After surface sterilisation, explants were washed three times with sterile distilled water (SDW) or until all traces of the surface sterilant and detergent were removed. Sterilisation was done in 100 ml sterilin jars.

Explants were removed from sterilised tissues using a stereomicroscope, sterile forceps, scalpel blade or hypodermic needle as appropriate and were generally dissected on sterile filter paper (Whatman No.1) to remove excess water.

Table 1 - Type and sterilisation of plant material

Explant	Source material	Sterilisation	
		con.^{n} of NaOCl (% v/v)	duration (min.)
1.leaf disc (cut with a 5 mm dia--meter cork borer)	leaf lobes on either side of the middle lobe	5	5
2.leaf lobe (young)	leaf adjacent to apical shoot	5	5
3.petiole section (1cm)	petioles from 3rd and 4th leaves	10	10
4.shoot tip	tip of stem	5	5
5.stem node (2cm)	stem (young, green)	10	10

All explants except for stem nodes were cultured in MS_{20} supplemented with various growth regulators to initiate friable and organogenic callus,as shown in Table 2.

Stem nodes were placed 5 each in 250ml flasks containing 50ml of liquid MS_{20} and 2 each in solid MS_{20} in 100ml Sterilin jars.

Table 2 - Type of explant and media used

Type of explant	Growth regulators + MS_{20}
leaf discs	0-22.1mg/l 2,4-D and BAP
petioles	(i) 0-22.1mg/l 2,4-D and BAP (ii) 0.01, 0.1, 1, 2, 4, 6, 8 mg/l 2,4-D (iii) 0.01, 0.1, 1, 2 mg/l IBA
shoot tip	0.1 mg/l BAP, 0.1 mg/l GA_3
leaf lobe	2.0, 4.0, 6.0, 8.0 mg/l 2,4-D

c).Culture incubation conditions

Explants cultured on solid media were incubated at 25±1°C on shelves with vertical or horizontal illumination provided by warm white 65-80 watt fluorescent lamps in a culture room with a 16h photoperiod (light intensity 45µE $m^{-2} sec^{-1}$).

Liquid cultures were incubated under the above temperature and light levels in a rotary shaker at 100rpm.

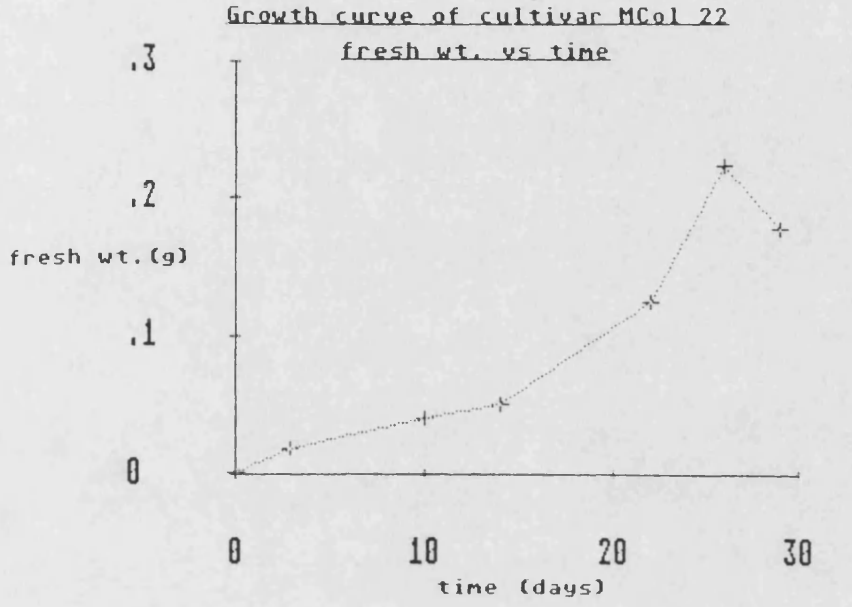
3.Establishment of callus cultures

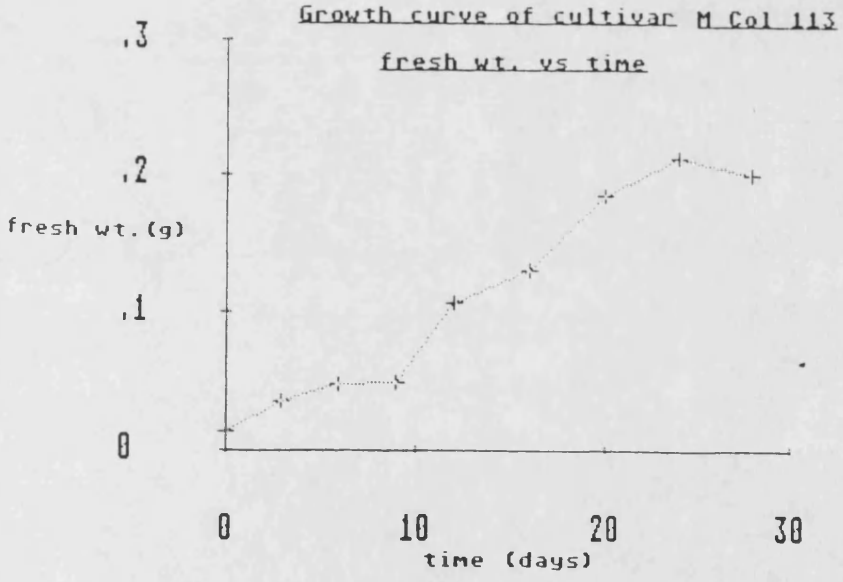
1cm long petiole segments were cultured as described earlier in MS medium with 2% sucrose and 0.6% agar supplemented with 4 mg/l 2,4-D. After 1 month, 0.5cm^3 friable callus pieces were subcultured onto fresh medium. These were further incubated for 2 weeks and used for experiments when necessary.

4.Establishment of suspension cultures from callus

Cell suspension cultures were initiated by dividing ca 5g of above callus and transferring to 50ml of liquid MS medium with 2% sucrose supplemented with 4 mg/l 2,4-D in screw top 250 ml flasks. The flasks were then incubated at 25°C in an illuminated gyratory shaker (light intensity 135 $\mu E\ m^{-2} sec^{-1}$) at 150 rpm. The first subculture was done after 4 weeks when the suspensions became turbid. The flasks were agitated and allowed to stand for 1 min. allowing the large clumps to settle at the bottom. 10 ml were withdrawn and added to 50 ml of fresh medium. The growth of these cultures was studied by estimating the fresh weight at various intervals. The subsequent sub cultures were done when the cultures reached late linear phase which was after about 25 days for cultivar MCol 113 and 28 days for cultivar M Col 22 as shown in Fig. 1.

Fig. 1- Growth curves of suspension cultures of cassava cultivars MCol 22 and MCl 113.





Growth was determined by measuring the fresh weight of cells over a 28 day time period.

5.Establishment of plantlets

Nodes were subcultured from the stems that developed from stem nodes cultured in liquid MS_{20}. One node each was placed in 50ml sterilin jars containing solid MS_{20} medium and incubated in a growth room under the conditions described earlier.

6.Bacterial isolates

Nine isolates of the bacterium Xanthomonas campestris pv manihotis were used in this study.

Details of the bacterial strains are as follows-

Isolate	Year of isolation	Place of isolation
2444	1970	Colombia
2967	1976	Brazil
3059	1973	Zaire
3066	1976	Nigeria
3161	1976	Cameroon
3172	1977	Zaire
3191	1973	Brazil
3194	1978	Niger
CIAT 1222	1984	Colombia

a).Culture media

The complete liquid medium - Nutrient Yeast Glycerol

Broth (NYGB) for Xanthomonas (Turner et al.,1984) containing 5g bacteriological peptone (Oxoid), 3g yeast extract (Oxoid), 20g glycerol, 1l water was used. Complete solid medium (NYGA) was prepared by adding 14g agar (Oxoid) per litre of NYGB. Sterilisation of media was by autoclaving at 121°C and a pressure of 1.4 Bars for 15 miniutes.

Bacterial growth in NYGB, Minimal medium A (Miller, 1972), Minimal medium B (modification of Dekker & Candy, 1979), modified Watanabe medium (Watanabe, 1963) (see Appendix I for details) and also under restricted culture conditions was monitored. The restricted culture conditions were obtained by adding concentrated nutrients into diffusion capsules (Cooper & Wood, 1975).

b).Maintenance and storage of bacterial cultures

Bacterial strains were stored for short periods on NYGA plates at 4°C or for longer periods as frozen suspensions in NYGB containing 20% (v/v) sterile glycerol at -70°C.

When required, cultures were thawed and streaked onto fresh NYGA plates.

c).Measurement of bacterial growth

Overnight broth cultures of all 8 strains were grown in NYGB at 30°C. 1 ml from each of these was added to

100ml of fresh NYGB and OD measurements at 600nm . were obtained with a PU 8650 visible spectrophotometer at hourly intervals. In addition, 1 ml samples were taken approximately every two hours, serially diluted in SDW and lawned onto NYGA plates. The plates were incubated at 30°C for 2 days prior to counting the bacterial colonies that had grown. This enabled a calibration curve of mean viable cell counts against optical density to be plotted.

7.Preparation of bacterial inoculum

The inoculum was prepared by incubating the bacterium overnight in 50ml of NYGB in 250ml Erlenmeyer flasks in a reciprocal shaker at 30°C. The bacterial cells were spun down by centrifugation (13000g for 15 mins.) and washed once in SDW. The resulting pellet was resuspended in SDW and adjusted turbidometrically at 600nm to give the required final concentration.

8.Preparation of culture supernatants

Cell free culture fluids were obtained by centrifuging the bacterial cultures at 13000g for 15 minutes and filter sterilising the supernatant using a Millipore filter with pore size 0.2µm.

9. Methods of inoculation of plants

The following methods of inoculation were tested for disease development.

a).spraying till runoff with the bacterial suspension (with 2 drops of Tween 80 added)

b).stabbing the stem at 3rd and 4th leaf axils with a needle dipped in the bacterial suspension

c).injection of 1ml of bacterial suspension to -

i)the base of the stem

ii)the base of the petiole of the fourth leaf

iii)the tip of the petiole of the fourth leaf

iv)the stem at 3rd and 4th leaf axils

d).clipping leaf lobes in half with scissors dipped in the bacterial suspension (CIAT,1975).

e).infiltrating 0.5ml of bacterial suspension into the abaxial surface of the leaf using a sterile syringe.

Plants were evaluated for symptom development when inoculated with Xcm isolates. The bacterial inocula were prepared as mentioned previously to give a final concentration of $1x10^8$ cfu/ml. There were four replicate plants for each treatment. Control plants were treated similarly with SDW.

All plants were kept in a Saxcil growth chamber (Saxton Sax Air Ltd.) with 30°C daytime and 25°C night time temperature, 12h photoperiod and 70-90% relative humidity. Plants were observed daily for symptom development.

10.Viability assessment of plant cells

(a)Suspension cultured cells

The viability of suspension cultured cells was assessed by staining with fluorescien diacetate (FDA) stain and viewing under an Olympus BH 2 fluorescence microscope with epiillumination. The exciting light wave length was in the blue region (490nm). The cells were considered as viable if they fluoresced brightly. Dead cells fluoresced weakly or did not fluoresce at all.

b).Stem tissue

Cell viability was demonstrated with the vital stain, neutral red (Tribe, 1955). Disks (1.5mm thick) were obtained from young cassava stems(2mth old plants,the upper 4cm of stem used) with a hand microtome. Disks were washed in several changes of stirred distilled water to remove cytoplasmic contamination from cut edges, then drained and surface dried quickly with tissue paper before adding 10 discs per ml to reaction mixtures containing bacterial culture fluids (see Results, Section III,B,3) in 5cm petri dishes; 10 disks were added per ml of the following reaction mixtures.

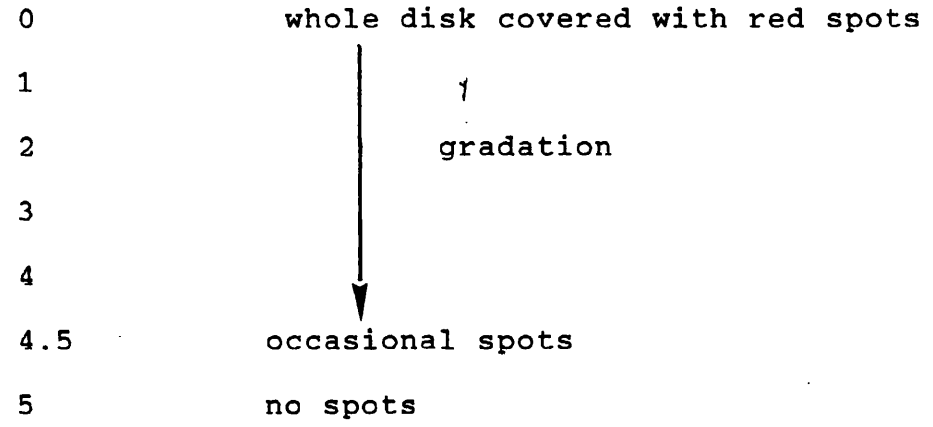
At intervals, 5 disks from each mixture were removed and transferred to a watch glass containing c. 2ml of the following solution:

85ml $MKNO_3$

10ml 0.1% neutral red (this solution was freshly prepared as the neutral red slowly precipitates in alkaline solution)

5ml 0.1M phosphate buffer p^H 7.5

The disks became plasmolysed and accumulated neutral red in vacuoles. After 20 min the neutral red solution was replaced by 0.9M KNO_3 solution buffered with 10ml 0.1M phosphate buffer for each 90ml of KNO_3 solution. Vacuoles of plasmolyzed protoplasts retaining the stain was considered to indicate living cells. The disks were compared under a low power microscope and results were recorded by a number (Tribe,1955), the Neutral Red Index (NRI) as follows:



11. Electrolyte leakage

(a) Infected leaves (Brisset & Paulin, 1991)

6mm diameter leaf discs were cut with a cork borer (No. 3) from surface sterilised (5% NaOCl, 5min) lobes of resistant and susceptible cassava leaves and tobacco leaves. 12 discs were transferred separately to suspensions ($1x10^8$ cfu/ml) of Xcm. isolates 2967 and 3194 and Erwinia amylovora isolate 1430 or 0.5mM MES buffer+0.5mM $CaCl_2$ (p^H 5.7) and infiltrated under reduced pressure. 4 disks each were transferred into 1ml of sterile 0.5mM MES+0.5mM $CaCl_2$ adjusted to p^H 5.7. There were 3 replicates for each treatment. Disks were incubated at 25°C with continuous stirring and artificial illumination.

The conductivity of the incubation medium was measured over a time period.

(b) Calli

Batches of friable callus tissue were weighed aseptically and transferred 1g each into 5cm petri dishes containing solid MS medium. 10µl of the calibrated bacterial inoculum was pipetted over the surface of the callus tissue and the dishes incubated in a growth room 25°C, 16h daylength and a light intensity of 45 $\mu Em^{-2} sec^{-1}$.

Dishes of tissue were removed from incubation at

time intervals, and the callus fragments carefully transferred to a boiling tube containing 10ml deionised water. The tubes were shaken for 10 seconds and the conductivity of the ambient solution measured using a PTI-18 digital conductivity meter. Conductivity was remeasured after incubation of the tubes for 10 mins at room temperature. Results were expressed as the increase in conductivity of the ambient solution over a 10 min period.

12. Assays for toxicity

Three assays were used to test for toxicity of cell free fluids from Xcm cultures.

Assay 1). Leaf infiltration - third and fourth leaves of 2mth old susceptible cassava cultivars M Col 22 or 113 were infiltrated in the abaxial surface with 1ml of the appropriate culture fluid using a sterile syringe without a needle. Control plants were infiltrated in the same manner with the uninoculated medium.

These plants were maintained in a Saxcil growth chamber at a relative humidity of 90%, 12h daylength and 30°C daytime and 25°C night time temperatures and observed for any visual effects caused by the culture fluids.

Assay 2). Effect on the viability of suspension cultured

plant cells - 20d old cells of susceptible cassava cultivar M Col 113 maintained in Murashige and Skoog (MS) medium supplemented with 20g/l sucrose and 4mg/l 2,4-D were centrifuged (2000g, 10 mins.) and added to 10ml of 1/10 strength MS medium at a concentration of 1% v/v. 10ml of the cell free culture fluid was added to this and the flasks were maintained at 25°C in an illuminated gyratory shaker (speed 150rpm). The viability of the cells were determined over a time period using fluorescein diacetate.

The controls consisted of plant cells suspended at 1% v/v in 10 ml of 1/10 MS and 10ml of uninoculated medium.

Assay 3).Ion leakage from stem and leaf discs- discs were obtained from the stems or leaves of the susceptible cassava cultivar M Col 113. The stems were cut transversely into 1.5mm thick slices using a hand microtome. Discs of 6mm diameter were cut from leaves using a flame sterilised cork borer.

Discs were placed in batches of 50 in 100ml of cell free culture fluid in 250ml flasks and incubated at 28°C in a gyratory shaker (speed 150 rpm). 1ml aliquots were removed at various time intervals and the K^+ concentration was determined using a flame photometer (Corning 400).

13. Reisolation of bacteria from cassava tissues

(a) Qualitative method

Plants of susceptible cassava cultivar M Col 22 were inoculated with Xcm isolate 2967 using the leaf infiltration and stem injection methods (Materials & Methods 9). The control plants were similarly inoculated with SDW.

The plants were maintained in a Saxcil growth chamber with 12h photoperiod, 30°C daytime and 25°C night time temperature, and 90-100% RH.

Four plants were sampled every other day for each method of inoculation.

Leaf tissues from infected plants were sampled as follows-

The leaves were surface sterilised for 5 mins with 5% NaOCl and the petioles for 10 mins with 10% NaOCl. Three 6mm discs were cut from each leaf lobe and 2mm sections from every 2cm of the petiole.

Stem tissues from infected plants were sampled as follows-

All the stems and petioles sampled were surface sterilised for 10 min with 10% NaOCl ; leaves in the manner mentioned earlier. 2mm stem sections were obtained from the point of inoculation and from every 3cm of the stem both proximal and distal from the point

of inoculation. All sections were plated on NYGA and incubated at 28°C.

The bacterial colonies that developed from some of the sections placed in NYGA were subcultured and reinoculated into healthy cassava plants and observed for symptom development.

(b)Quantitative method

The third leaf of 2 month old plants of cultivars MCol 22 and MNGA 1 were inoculated with a suspension of Xcm isolate 2967 using two methods of inoculation (Results, Section II,3(ii).

Inoculated leaves were surface sterilized for 5mins with 5% NaOCl and washed with SDW to remove all traces of the surface steriliant.

Leaf discs (6mm diameter) from 5 replicate leaves were cut with a sterile cork borer every other day - i) from the inoculated lobe and the two lobes on either side ii).from 1/2cm and 6cm from the infected surface depending on the method of inoculation.

All discs were comminuted in 0.5M MES (p^H 5.7), serially diluted and plated on NYGA to estimate the bacterial numbers that developed.

14. Production of cell wall-degrading enzymes

Culture conditions and C sources

Bacterial isolates suspended in SDW and adjusted turbidometrically to give $1x10^8$ cfu/ml then were added to 100ml of the various media to give a final concentration of $1x10^7$ cfu/ml. Various C sources were added to the basal medium (minimal medium B). These were- 1).glucose (Sigma)

2).0.25% xylan (Birchwood, Sigma)

3).0.25% sodium polypectate (NaPP) (Sigma)

4).0.5% cassava cell walls

The cassava cell walls were obtained from cassava stems and petioles following the method used by Karr & Albersheim (1970).

All media were autoclaved for 15min at 121°C and the final p^H was adjusted to 6.8.

All flasks were maintained in shake culture (120rpm) at 28°C and the bacterial growth was monitored by dilution plate counts for cultures with cell wall added as C source and by absorbance measurements at 600nm for the others.

The culture supernatants were obtained at two stages of bacterial growth, dialysed overnight at 4°C against large volumes of distilled water (P^H 7) and stored on ice at 4°C.

15.Enzyme Assays

There were 3 replicates for each assay at each sampling time.

a).Pectate lyase assay (Dow et al., 1987)

PGL was assayed spectrophotometrically at 30°C by measuring the changes in absorbance at 235nm using a Shimadzu UV-260 spectrophotometer. The assay mixture contained 0.7ml 0.25% w/v NaPP in 0.05M Tris-HCl buffer (p^H 9), 1mM $CaCl_2$, and 0.3ml of the culture supernatant. By using the molar extinction coefficient of the unsaturated bonds produced (4600) (Nagel & Anderson, 1965), the OD can be expressed as umol unsaturated galacturonic acid (UGALA)/ml/min.

b).Pectin lyase assay

PL was assayed spectrophotometrically at 30°C by measuring the changes in absorbance at 240nm. The assay mixture was as for PGL but with pectin (Sigma) as substrate.

c).Endo-polygalacturonase (endo-PG) (Cooper & Wood,1975)

Activity was determined by viscometric assay. Activities are expressed as relative viscometric units (RUV), defined as 10^3 x reciprocal of time (min) for 50% decrease in relative viscosity (t_{50}) of a reaction mixture of 8ml solution of substrate (1% w/v NaPP),

buffered at pH 5 (0.1M citrate) and 2ml enzyme solution contained in Cannon-Fenske viscometers (size 200) at 25±1°C.

d).Pectin methyl esterase (PME)

PME was assayed by the continuous titration method by Bateman (1963). Reaction mixtures contained 20ml of 1.2% citrus pectin N.F.. 2ml of 1N NaCl and 3ml of culture supernatant left for 30min at 30°C and titrated with 0.01N NaOH to a faint colour, a few drops of $KMNO_4$ having been added as an indicator. PME activity is expressed in milliequalent of pectinic acid produced/ml of enzyme h.

e).Protease (Movahedi, 1990).

Protease activity was assayed spectrophotometrically by measuring the release of red dye from the protease substrate azocasein (3%) at pH 5.0 and 8.0.Azocasein is casien with an azo dye attached. As the peptide linkages of the casein are hydrolysed, the bound dye is solubilised and the concentration of the solubilised dye is determined by measuring the absorbance of enzyme reaction mixtures at 366nm.

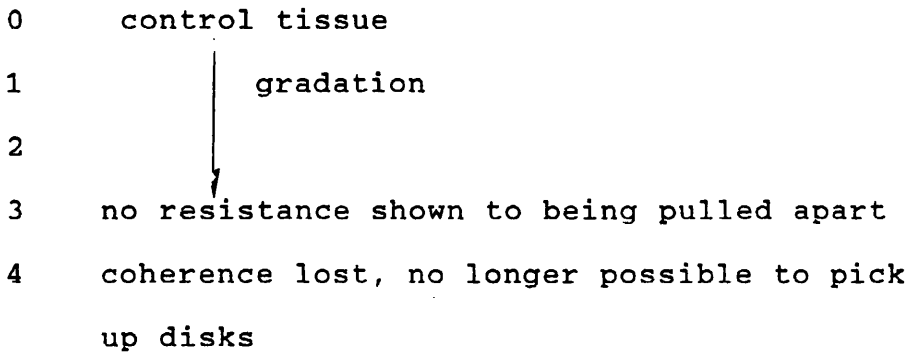
Enzyme reaction mixtures contained 1ml 3% azocasein, 0.35ml of 50mM acetate buffer pH 5.0 or Tris-HCl buffer at pH 8.0 and 0.05ml sample. After incubation at 30°C for

30min, the reaction was stopped by the addition of 0.5ml 20% TCA. The solutions were centrifuged (1000g for 10min) and the absorbance of the supernatant read at 366nm.

16. Maceration of cassava tissue by endo-PL

Macerating activity was estimated by testing the loss of coherence of stem tissue disks prepared as for the neutral red assay. 5 disks were removed from reaction mixtures (see Results, Section III,b,3) and subjected to a standard stress between two dissecting needles.

Results were recorded by a number, the Maceration Index (MI) as follows:



17. Extraction and detection of enzymes from cassava tissue (Cooper & Wood, 1980)

Tissue from infected and control leaves was comminuted in liquid N_2 to a fine powder. This was extracted in 0.025M phosphate buffer (pH 6) containing 0.2M NaCl (to desorb enzymes from cell walls) 1mM dethiothreitol and 5% insoluble polyvinyl polypyrrolidone

(PVP, BDH) (to prevent oxidation and adsorb phenols respectively) at 1g tissue per 8ml buffer. Tissue was extracted on ice by stirring and after 5 min strained through 2 layers of muslin and centifuged (23000g, 15min, 4°C). The clarified extract was dialysed (overnight, 4°C) with a large volume of distilled water (p^H 7). All samples were concentrated at 4°C against PEG 20,000 and assayed for PGL activity using the 235 assay.

18.a).Flatbed isoelectric focusing

Isoelectric focusing of PGL was performed on LKB 2117 Multiphor apparatus in ready prepared (Pharmacia) ultrathin (245x110x1mm) polyacrylamide gels. (PAG plates) which contain carrier ampholytes to give a 3.5-9.5 pH range. The gel was fixed to the cooling plate and the electrode wicks for the anode and cathode were soaked for several minutes in 1M H_3PO_4 and 1M NaOH respectively. 20µl samples of the enzyme with 1% glycine added to reduce the salt level (the enzyme was dialysed overnight against large volumes of distilled water at 4°C and concentrated x10 against PEG) were applied to the gel in segments (ca. 10mm) of chromatography paper. The gel was electrofocused for 1.5h at maximum voltage (340V) and current (72amp.) and 25W power; sample application paper was removed after 30min after electrofocusing had begun. The pH gradient was determined with an isoelectric focusing calibration kit (Pharmacia).

b).Activity stain overlay technique for detecting PGL

(Reid & Collmer, 1985).

An overlay of 4mm thickness containing 1% sodium polypectate (NaPP), 50mM Tris-HCl (pH 8.5), 1.5mM $CaCl_2$ and 1% agarose (Sigma) was prepared on gel support film. The casting apparatus was heated to 50°C before the pectate-agarose mixture at 95°C was pipetted. The cooled pectate-agarose overlay was pressed against the IEF gel and incubated at 30°C for 3h. The agarose overlay was then placed in 0.05% ruthenium red for 30min and rinsed in several changes of distilled water. Clear bands in a red background indicated PGL activity.

19.Total and free amino acid analysis of cassava leaves

(Spackman et al., 1958; Moore & Stein, 1951)

Amino acid analysis was kindly carried out by Ms V. Plumb (Natural Resources Institute, Kent, UK). (See Appendix III for full details of methods).

Samples from infected and control leaves were instantly preserved in liquid N_2 and freeze dried for storage.

For the analysis of all amino acids except for methionine and cysteine, 100mg of dried leaf tissue was hydrolysed with 100ml of 6N HCl and filtered. The filtrate was dried at 40°C by rotary evaporation, washed

with deionised water to remove the acid and the residue dissolved in pH 2.2 citrate diluting buffer. The solution was then loaded onto the amino acid analyser for separation.

For the analysis for methionine and cysteine, oxidation with 10ml of performic acid (45ml of 88% formic acid + 5ml 30% w/v H_2O_2) was done prior to carrying out the above procedure. Samples spiked with methionine gave 26.5% recovery.

20.Transmission Electron Microscopy

a). Plant inoculation

Two month old plants of susceptible cassava cultivar MCol 22 were inoculated separately with a suspension of Xcm isolate 2967 using leaf infiltration or stem injection methods (see Materials & Methods 9 and Results and Discussion Section IV for details). The control plants were treated similarly with SDW.

b).Preparation of tissue for electron microscopy

i).Leaves

Infected control samples (ca.2mm) were cut from the appropriate areas (see Results and Discussion Section IV for details) and fixed in 4% Glutaraldehyde and 1% Acrolein in 0.05M PIPES buffer (pH 8) at 4°C for 16h. Vacuum infiltration of fixative was not carried out to avoid the disruption of bacterial colonies within

intercellular spaces (Smith & Mansfield, 1982). The fixative was then removed by washing in 0.2M PIPES buffer (pH 8, osmolarity 823mosm approx.) containing 0.2M sucrose for 45min at 4°C. Postfixation was carried out in 1% Osmium tetroxide in 0.15M PIPES (pH 7) containing 0.2M sucrose for 1h at 4°C. The tissues were then washed in several changes of distilled water over a 30min period at 4°C to remove the postfixative.

ii). **Stem**

Infected and control samples (ca.2mm^2) were cut from the appropriate areas (see Results and Discussion Section IV for details) and fixed at 4°C in 2.5% glutaraldehyde and 1% acrolein in 0.05M sodium cacodylate buffer (pH 8,approx. 450mosm.) for 16h. Vacuum infiltration was not carried out to avoid disruption of bacterial colonies. The fixative was removed by washing in 0.1M sodium cacodylate buffer (pH 8) containing 0.15M sucrose at 4°C for 45 min and postfixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7) containing 0.15M sucrose for 1h at 4°C. The tissues were then washed in several changes of distilled water over a 30min period.

Both stem and leaf samples were then processed in the same manner as follows:

The tissues were dehydrated through a graded acetone series, with4-5 changes for each step (30min in 20%, 30%,

40%; 20min in 50%; 30min in 60%; 25min in 70%; 30min in 80%; 45min in 90%; 30min in 95%; 30min in 100% and 45 min in 100% dry acetone BDH, Analar Grade) at 4°C.

The tissues were then infiltrated with an epoxy resin (TAAB premix resin) in a rotator (TAAB) by slowly increasing the proportion of resin to acetone in different solutions over a 32h period (acetone:resin-3:1, overnight, 1:1 8h, 1:3 overnight). Tissues were then left for 1h in pure resin without accelerator in an oven at 60°C; this enabled the penetration of resin into the tissue. Samples were removed from the oven, cooled to room temperature and placed in fresh resin with added accelerator for 6h at room temperature. The tissue was also subjected to vacuum infiltration for a few minutes at this step for better penetration. After 6h the tissues were placed in a freezer for one month after which the tissues were removed and warmed to room temperature and placed in fresh complete resin for 16h at room temperature.Finally, both leaf and stem pieces were placed in mounting block pieces filled with pure resin and polymerised at 60°C for 48h. Sections of the resulting block containing the tissues were trimmed and ultrathin sections cut with a Reichert OMV 3 ultramicrotome fitted with a diamond knife. Sections were collected on copper grids, double stained with 7% aqueous uranyl acetate for 8-10min and lead citrate (Reynolds,

1963) for 4-5min and viewed under a JOEL 1200EX transmission electron microscope operating at 80kv.

RESULTS AND DISCUSSION

SECTION I - USE OF A TISSUE CULTURE SYSTEM TO INVESTIGATE THE PATHOGENICITY OF Xcm TOWARDS CASSAVA

Various cassava tissues were cultured on a range of media with a view of developing an *in vitro* system(s) to study critically the interaction between *Xcm* and cassava. An *in vitro* system that shows a similar reaction to the whole plants when inoculated with the bacterium could be of great use as it should facilitate critical microscopical, biochemical and molecular approaches at cellular level under conditions of synchronous infection and which can be manipulated and controlled. Such host cells may also provide a siutable bioassay for bacterial toxins and under suitable selection pressure surviving cells may provide a source of novel disease resistant lines.

1. Tissue culture of cassava

Several tissues excised from cassava plants were cultured on various solid media. As Table 5 indicates, the majority of treatments resulted in initiation of undifferentiated friable callus and some in adventitious root formation. The friable calli that developed differed only slightly in their compactness and colour. Young leaf lobes produced callus at all concentrations of

2,4-D tested, i.e. 2, 4, 6, and 8 mg/l. Petiole sections cultured on 0.01, 0.1 and 1.0mg/l 2,4-D failed to produce callus, but those cultured on 2, 4, 6,and 8 mg/l showed callus proliferation. Petiole sections cultured on 0.01, 0.1, 1.0 and 2.0 mg/l IBA failed to produce callus, but produced roots at 1.0 and 2.0 mg/l concentrations.

Culture of stem node explants on semi-solid MS_{20} produced shoots and roots and the time taken was almost the same for all cassava cultivars tested except for MCol 22 which produced shoots and roots within 3 weeks as shown in Table 3.

Table 3 - Root and shoot production from stem node explants cultured in solid MS_{20}

cultivar	Time for shoot prod.[a] (weeks)	Time for shoot prod.[a] (weeks)
MNGA 1	4	5
MCol 22	3	3
MCol 113	4	5
MVen 77	4	5

Nodes cultured in liquid MS_{20} produced shoots and roots both of which differed in length to those produced on the semi-solid medium (Table 4). The initial "breaking of buds" took place simultaneously in both types of cultures i.e. after 6 days, but stem elongation in

the liquid cultures after 15 days was 2-5 times more efficient than in the solid cultures (Table 4, Plate 1).

Table 4 Lengths of shoots produced in solid and liquid MS_{20} after 15 days incubation

cultivar	length (cm) semi-solid MS_{20}	liquid MS_{20}
MCol 22	3.5	10.5
MNGA 1	2.0	9.0
MVen 77	2.5	7.0

(Mean values of lengths of stems in 8 vessels, 5 stems each in liquid media and 2 each in semi-solid).

Nodes from these shoots that developed in both types of culture were subcultured onto semi-solid MS_{20}, they, in turn, developed into plantlets within 4 weeks.

Table 5 - Response of cassava explants to various media

explant	medium ($+MS_{20}$)	callus form.[a]	time (weeks)	root form.[a]	time (weeks)
young	2,4-D- 2mg/l	+	4	-	-
leaf	4mg/l	++	4	-	-
lobes	6mg/l	++	4	-	-
(.25mm)	8mg/l	++	4	-	-
petiole	2,4-D-0.01mg/l	-	-	-	-
sections	0.1 mg/l	-	-	-	-
(1cm)	1.0 mg/l	-	-	-	-
	2.0 mg/l	+	4	-	-
	4.0 mg/l	++	4	-	-
	6.0 mg/l	++	4	-	-
	8.0 mg/l	++	4	-	-
	IBA-0.01 mg/l	-	-	-	-
	0.1 mg/l	-	-	-	-
	1.0 mg/l	-	-	+	5
	2.0 mg/l	-	-	+	5

Relative callus form.[a] (quantity): - none + low ++ high

Callus was also initiated from various explants using a combination of 0, 0.022, 0.22, 2.2 and 22.0mg/l 2,4-D and BAP. The results are shown in Tables 6 a,b and c.

Friable callus formation from petiole pieces of both cultivars was the best on 2,4-D and BAP concentrations 2.21mg/l, 22.1mg/l; 0.22mg/l, 2.21mg/l; 22.1, 0.22mg/l. (Plate 2).

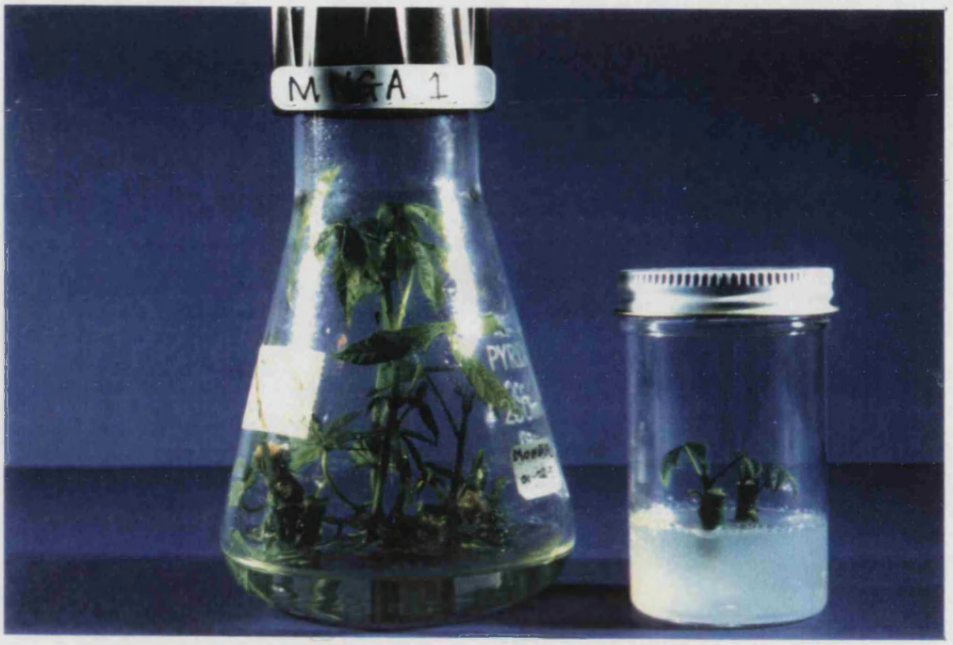
Callus was not initiated from 5 mm diameter leaf discs of cultivar MCol 22 cultured in the absence of 2,4-D or BAP and also on 0.022mg/l concentration of both.Friable callus formation was best on 2,4-D and BAP concentration combinations 2.2 and 0.022mg/l, 2.2 and 0.22mg/l and 22.0 and 0.22mg/l.

Table 6d shows the effect of the combination of NAA and BAP at concentrations 0, 2, 4 and 8 mg/l on petiole sections (1 cm) of cultivar MNGA 1. BAP concentrations 0,2,4 and 8mg/l failed to produce callus in the absence of NAA. NAA concentrations 2,4 and 8mg/l produced a small amount of callus and roots even in the absence of BAP. The other combinations produced prolific friable callus.

Plate 1- Shoot production of cassava cultivar MNGA 1 in solid and liquid MS_{20} after 15 days incubation.

Plate 2- Friable callus formation from 0.5cm petiole segments of cassava cutivar MCol 22 on a range of combinations of 2,4-D (0.022-22.1mg/l) and BAP (0.022-22.5mg/l) after four weeks incubation.

1



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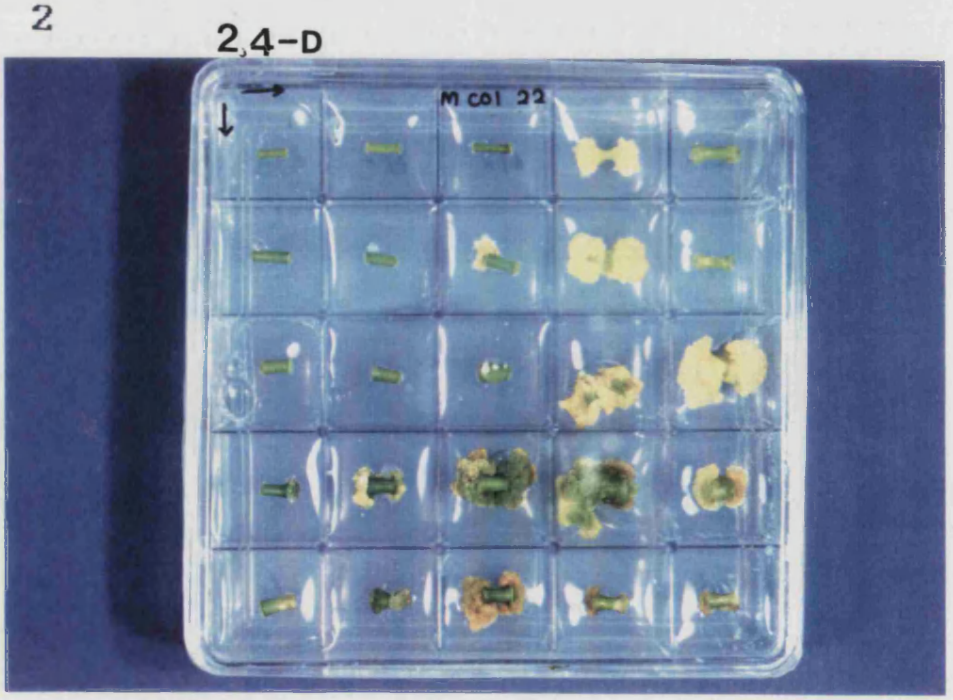


Table 6 a,b and c- The effect of combinations of 2,4-D and BAP on various explants.

Table 6a- Petiole sections of cultivar MNGA 1 (0.5 cm pieces).

		2,4-D(mg/l)				
		0	0.022	0.22	2.2	22.0
BAP(mg/l)	0	c1	c1	c1	c2	c3
	0.022	c1	c1	c1	c3	c2
	0.22	c1	c1	c2	c3	c2
	2.2	c1	c1	c2	c3	c3
	22.0	c1	c1	c1	c3	c3

Table 6b- Petiole sections of cultivar MCol 22 (0.5 cm pieces)

		2,4-D (mg/l)				
		0	0.022	0.22	2.2	22.0
BAP (mg/l)	0	c1	c1	c1	c2	c1
	0.022	c1	c1	c1	c2	c1
	0.22	c1	c1	c1	c3	c2
	2.2	c1	c1	c3	c3	c3
	22.0	c1	c2	c2	c2	c3

Table 6c- Leaf discs (5 mm diameter) of cultivar MCol 22

BAP (mg/l) \ 2,4-D (mg/l)	0	0.022	0.22	2.2	22.0
0	-	-	-	c1	c1
0.022	-	-	c1	c3	c1
0.22	-	c1	c1	c3	c3
2.2	-	c2	c2	c1	-
22.0	-	-	-	c2	-

Relative callus formation (quantity):- - = none
c1= low
c2= medium
c3= high

Table 6d- The effect of the combinations of NAA and BAP on petiole sections of cultivar MNGA 1

BAP (mg/l) \ NAA (mg/l)	0	2	4	8
0	-	c1r	c1r	c1r
2	-	c3	c3	c3
4	-	c2	c3	c3
8	-	c3	c2	c3

Relative callus formation (quantity):- - = none
c1= low
c2= medium
c3= high
cr= callus and roots

2. Use of callus cultures to study the interaction between Xcm and cassava

In an attempt to use *in vitro* host material to study the pathogenicity of *Xcm*. isolates and possible expression of host resistance *in vitro*, electrolyte leakage was measured from *Xcm* inoculated callus cultures of cassava cultivars MCol 22, MCol 113, MVen 77 and MNGA 1 as an indication of rate and extent of damage to host cells by the pathogen.

1g of friable callus tissue of each cultivar obtained in the manner described in Materials and Methods 2 and subcultured onto solid MS_{20} was inoculated by placing 10ul of *Xcm* isolates 3194 or 2967 (suspended in SDW to give a concentration of $1x10^8$ cfu/ml) on the surface of each piece. The controls consisted of calli treated similarly with SDW. Electrolyte leakage was determined as described in Materials and Methods 12b over a 48h period. Six replicate calli pieces were sampled for each treatment at each time. Tables 7 and 8 show that although the leakage of ions from inoculated callus pieces was generally higher than that of the controls, there was no consistent change with time in conductivity readings.

Table 7- Electrolyte leakage from callus tissue treated with Xcm isolate 3194 and SDW (control).

time(hr)	conductivity* (µs/cm)							
	113		22		77		MNGA1	
	c	t	c	t	c	t	c	t
0	0.018	0.02	0.013	0.014	0.012	0.013	0.010	0.011
2	0.019	0.015	0.014	0.017	0.013	0.015	0.014	0.015
6	0.016	0.017	0.013	0.015	0.013	0.015	0.016	0.017
12	0.015	0.016	0.014	0.015	0.016	0.017	0.014	0.015
16	0.014	0.015	0.013	0.20	0.015	0.016	0.015	0.015
24	0.010	0.017	0.024	0.027	0.031	0.035	0.014	0.017
36	0.012	0.014	0.015	0.016	0.020	0.020	0.015	0.018
48	0.010	0.013	0.012	0.015	0.018	0.019	0.014	0.016

113,22,77,MNGA 1- cassava cultivars tested (1g of callus for each replicate of each treatment)

c- control (callus treated with SDW)
t- treated (callus inoculated with 10ul Xcm isolate 3194)

* (mean values of six replicate readings at one time for each treatment).

Table 8- Electrolyte leakage from callus tissue treated with Xcm isolate 2967 and SDW (control).

time(hr)	conductivity* (µs/cm)							
	113		22		77		MNGA1	
	c	t	c	t	c	t	c	t
0	0.016	0.018	0.015	0.016	0.012	0.013	0.010	0.011
2	0.017	0.019	0.013	0.015	0.014	0.017	0.015	0.017
6	0.016	0.017	0.014	0.015	0.014	0.017	0.016	0.019
12	0.013	0.015	0.012	0.015	0.013	0.017	0.013	0.017
16	0.012	0.016	0.014	0.018	0.014	0.016	0.013	0.015
24	0.012	0.017	0.014	0.017	0.021	0.015	0.014	0.017
36	0.013	0.014	0.014	0.018	0.010	0.011	0.014	0.019
48	0.014	0.016	0.012	0.015	0.017	0.019	0.016	0.018

113,22,77,MNGA 1- cassava cultivars tested(1g of callus for each replicate of each treatment
c- control (callus treated with SDW)
t- treated (callus inoculated with 10ul Xcm isolate 2967)

* (mean values of six replicate readings at one time for each treatment).

3. Use of in vitro propagated plantlets for rating the pathogenicity of Xcm isolates

In vitro plantlets could be used as an early screen for possible disease resistance of potential somaclones; they should also facilitate the study of the host pathogen interaction. The possibility of using in vitro plantlets as a rapid screening method for the pathogenicity of Xcm isolates was investigated in the

present study. The possibility of multiplication of a number of genetically identical individuals is an added advantage for large scale screening programmes.

Plantlets regenerated from cassava stem nodes cultured first in liquid and subsequently on semi-solid MS_{20} were inoculated with Xcm isolates 2967, 2444, 3194 and CIAT 1222 to determine the possibility of using plantlets as host material to test the pathogenicity of Xcm and also to determine whether there was a differential response between susceptible and resistant cassava cultivars when inoculated in this manner.

Plantlets of cassava cultivars MCol 22 (susceptible) and MNGA 1 (resistant) regenerated in the manner described in Section I, 1 and maintained on semi-solid MS_{20} for 4 weeks, were stabbed in the stem below the fourth leaf axil with a sterile needle passed through single bacterial colonies. Inoculation was performed with Xcm isolates 2967, 2444, 3194 and CIAT 1222 maintained on NYGA plates and also with Erwinia amylovora isolate 1430 as a control. Another set of plantlets was stabbed with a flame sterilized needle dipped in SDW. The plantlets were observed for symptom production.

The first symptom appeared after 8 days on plantlets of both cultivars MCol 22 and MNGA 1 inoculated with Xcm isolate 2967 in the form of wilting of the leaf closest to the point of inoculation (Plate 3); whereas isolates

3194 and CIAT 1222 required 10 days and isolate 2444 required 12 days to produce the same symptom (Table 9). After 10 days, the plantlets inoculated with isolate 2967 showed severe chlorosis and complete wilt. The same effect was shown on plantlets inoculated with isolates 3194 and CIAT 1222 after 12 days and 2444 after 15 days. The plantlets treated with E. amylovora isolate 1430 and SDW remained healthy.

The system could be utilised to differentiate between the virulence of Xcm isolates and the symptoms and the time required for symptom production corelated with that of green house grown plants (Section II,2,ii). The plantlets of the resistant cultivar did not show a different response to that shown by the susceptible.

Table 9- Symptom production by Xcm isolates 2967, 3194, 2444 and CIAT 1222 on in vitro plantlets of cultivars MCol 22 and MNGA 1

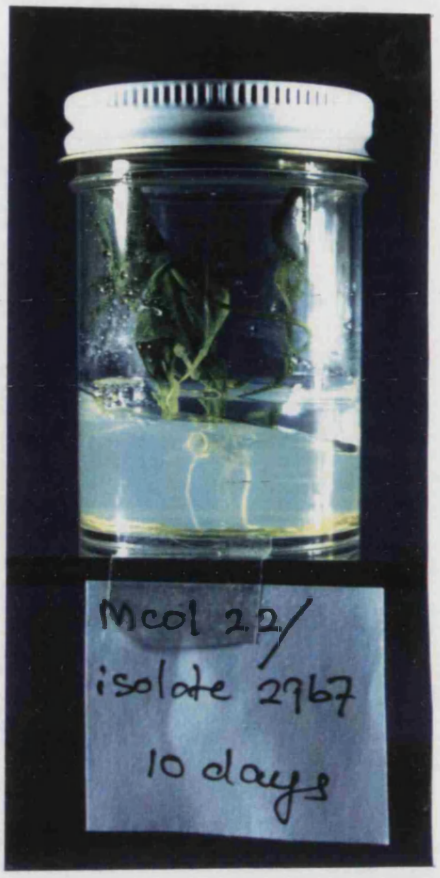
symptom	isolates 2967	3194	2444	1222
	time (days)			
wilting of leaf (at the point of inoculation)	8	10	12	10
chlorosis and complete wilt of the plant	10	12	15	12

There were 12 replicate plantlets for each treatment. These were incubated at 28°C, 16h photoperiod and 45µE $m^{-2} sec^{-1}$ irradiance.

Plate 3- Plantlet of cassava cultivar MCol 22 (susceptible) 10 days following inoculation with *Xcm* isolate 2967.

Plate 4- 20-day-old viable suspension cultured cells of cassava cultivar MCol 113 stained with FDA (magnification x400).

3



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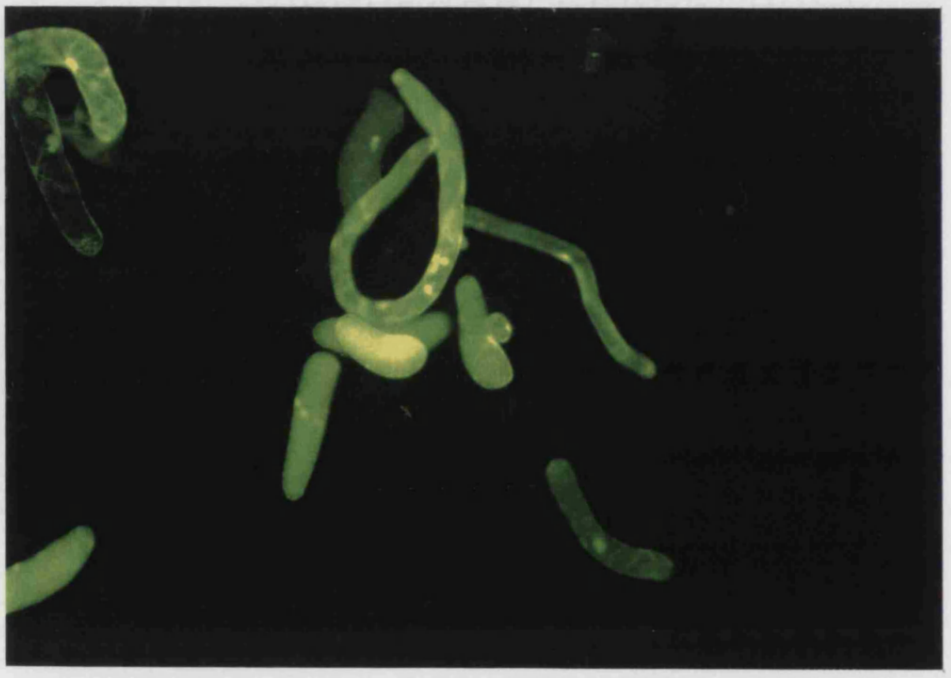


4.Use of suspension cultured cassava cells as an assay for bacterial pathogenicity

Cassava cell suspension cultures were examined for their potential for use as host material to study the effect of Xcm isolates or of their products.

(i).Viability and growth of bacteria and cassava cells in different suspending media

The purpose of this experiment was to find a suitable medium in which to suspend bacterial and host cells, in which host cell viability was retained at high levels in control treatments and bacterial growth was not prolific. If the medium was conducive only to plant cells or to bacterial cells, a true reflection of the interaction would not be shown.

a).Survival of cassava cells in different media

Cells from suspension cultures of cassava maintained as mentioned in Materials and Methods 4 were spun down at 2000g for 10 mins., the supernatant discarded, and the cells washed once with SDW. They were then added to 45 ml of the following media in screw top flasks to give a concentration of 1% v/v.

The following media were tested -

MS_{20}+4 mg/l 2,4-D MS

1/2 strength MS 1/10th strength MS,

PO_4 buffer (p^H=8), SDDW

The cultures were incubated in a gyratory shaker with a speed of 150 rpm at 25°C and a light intensity of 135μE $m^{-2}sec^{-1}$. There were 4 replicate flasks for each treatment.

Cell viability was evaluated daily using FDA. 400 cells were counted from each replicate flask at each one sampling time.

Table 10 -The mean percentage viability of suspension cultured cassava cells in a range of media

Day	% viable cells ± SE (mean values of 16 counts,100 cells per count)					
	MS_{20}+4mg/l 2,4-D	MS	1/2 MS	1/10 MS	PO_4 buf. (p^H=8)	SDDW
0	63±5.9	63±5.9	63±5.9	63±5.9	63±5.9	63±5.9
1	75±6.3	48±1.3	39±1.2	47±1.0	9±0.5	5±0.3
2	73±1.3	47±2.7	45±1.3	42±1.5	6±0.2	5±0.3
3	53±2.3	47±1.5	46±0.8	46±1.0	1±0.2	4±0.3
4	60±1.4	45±1.0	44±1.2	45±1.3	1±0.3	5±0.4
5	60±1.2	40±2.1	38±1.3	39±1.0		
7	53±1.4	26±0.6	24±0.7	24±0.5		

Cells were considered as viable if they fluoresced brightly when stained with FDA and observed under blue light (490nm) using a fluorescence microscope with epiillumination (Plate 4).

PO_4 buffer (p^H=8) proved to be toxic towards plant cells causing 91% mortality after 24 hours, and similarly only 5% of cells suspended in SDDW were viable after 24 hours. Therefore, both solutions were rejected. MS, 1/2 strength MS and 1/10 strength MS supported cell survival equally well with about 45% viability remaining after 3 days. Viability was only slightly less than for the cells suspended in MS with 2% sucrose and 4mg/l 2,4-D added which served as the original growth medium from which the cells were derived (Table 10).

b). Survival of bacteria cells

A suspension of Xcm isolate 3194 at $1x10^8$ cfu/ml was prepared as described in Materials and Methods 7 in SDW. 5ml of this suspension was added to 45ml of the following media in screwtop flasks to give a final concentration of $1x\ 10^7$ cfu/ml:

MS_{20} + 4mg/l 2,4-D, MS, 1/2 strength MS, $1/10^{th}$ strength MS

These flasks were incubated at 25°C in a gyratory shaker with a speed of 150 rpm. There were 4 replicate flasks for each treatment. Bacterial cell viability and growth was determined at daily intervals by serially diluting 1 ml aliquots from each flask and plating the appropriate dilutions on NYGA. The plates were incubated for 2 days at 30°C and the resulting numbers of colonies were estimated.

Table 11- The survival of bacterial isolate 3194 suspended in various solutions

days	Bacterial viability (c.f.u./ml(x10^5)			
	MS_{20}+4mg/l 2,4-D	MS	1/2 MS	1/10 MS
0	147.3	145.6	143.8	146.2
1	11.0	0.007	8.3	8.6
2	5.9	0.0	0.0	1.28
3	1.7	0.0	0.0	0.66
4	0.0	0.0	0.0	0.05

The results shown in Table 11 indicate that the bacterial cells survived in MS and 1/2 strength MS only up to 24 hours. However, the bacteria in MS medium with 2% sucrose and 4mg/l 2,4-D added and 1/10 strength MS retained viability up to 3 days.

Therefore 1/10 strength MS solution was selected as the most suitable solution to coculture cells in future experiments. It supported reasonable plant cell survival up to 5 days and bacterial growth up to 4 days. Furthermore, as this medium does not contain a C source, the probability of host cell death occurring as a result of accumulation of toxic metabolites from extensive bacterial growth is reduced.

(ii). Cassava cell bioassay for cell killing ability

These experiments were aimed at determining the validity of using inoculated suspension cultured cells to study pathogenesis. An obvious potential advantage of this sytem involving synchronous host-pathogen contact is the possibility of studing causes of initial cell damage. If successful, the system could be used for testing the possible involvement in pathogenicity and effects of bacterial toxins or enzymes. Also, if regeneration of plants from suspension cultured cells could be achieved, this could be a suitable system for selecting for disease resistant plants.

Cassava cells from suspensions of cultivar MCol 113 in lag (5day) and early (10day) and linear (20day) phase were used to examine the influence of age on response to Xcm. Fig. 1 (pg. 64) shows the growth curves of suspension cultured cassava cells determined by measuring the fresh weight of cells with time. The plant cells were spun down and resuspended in 45ml of 1/10 strength MS to give a 1% v/v concentration. 5ml of the bacterial suspension (Xcm isolates 3194 and 2967)) with $1x10^8$ cfu/ml was added to this to give a final concentration of $1x\ 10^7$ cfu/ml. The controls consisted of plant cells in 45ml of 1/10 strength MS with 5ml of SDW. There were 4 replicate flasks for each treatment. The flasks were incubated in a shaker at a speed of 150rpm at 25°C and a light intensity

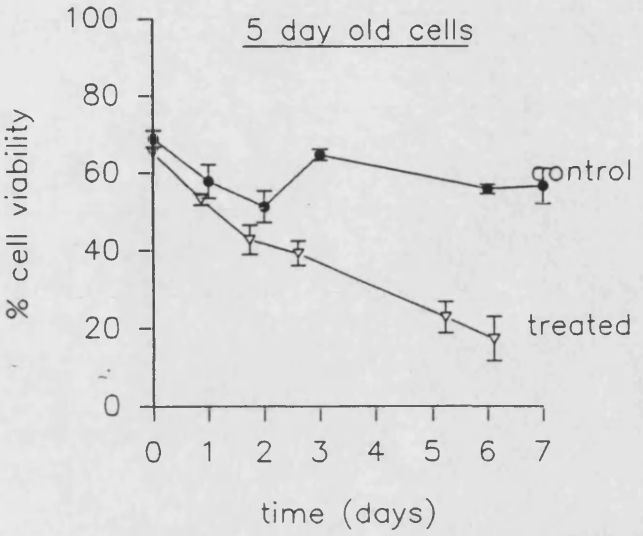
intensity of 135μE $m^{-1}sec^{-2}$. Cassava cell viability was determined at daily intervals using FDA.

Coculture with either isolate 3194 or 2967 resulted in death of cells of cultivar MCol 113, although the susceptibility of the cells varied with the age of host cells. Isolate 3194 caused 80% (when the initial viabilities are considered) host cell death by 7, 5 and 3.5 days in 5, 10 and 20day old suspension cultured cells respectively. At these times the viability of the controls was ca.70%, 80% and 90% (Fig. 2) and differed significantly ($P<0.05$) from cutures with Xcm.

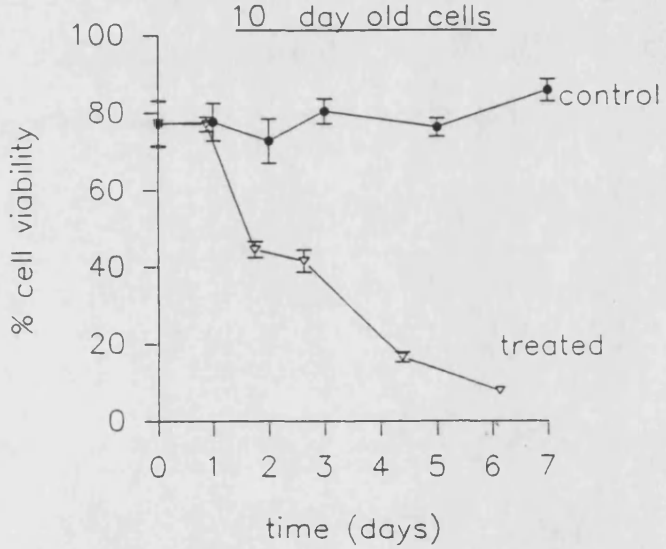
Isolate 2967 caused 80% cell mortality (when the initial viability is considered) by 7 and 3.5 days in 10 and 20 day old suspension cultured cells repectively thus showing a significant difference ($P<0.05$) from the controls 90% and 95% viability at these times (Fig. 3).

The bacterial population decreased or remained stable until the onset of plant cell mortality after which the numbers increased, presumably as a result of the nutrients released from damaged suspension cultures.

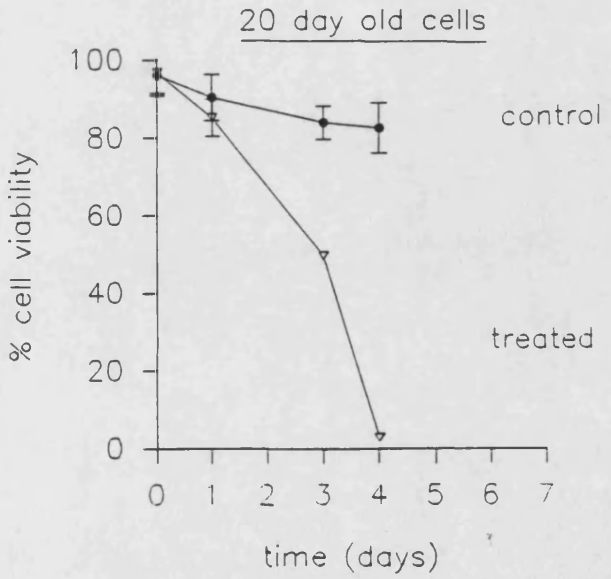
Fig. **2** –% cell viability of cells from 5, 10 and 20 day old suspension cultured cells of cultivar MCol 113 inoculated with Xcm isolate 3194



time (days)	bacterial no.(cfu/ml
0	6.4×10^7
1	1.8×10^5
2	9.0×10^5
3	1.5×10^4
6	4.2×10^6
7	8.0×10^5



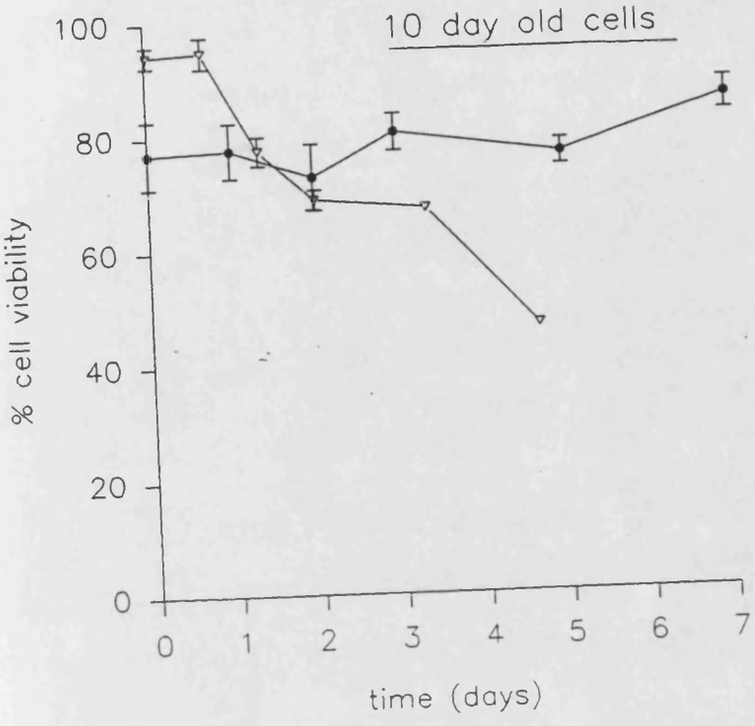
time (days)	bacterial no.s(cfu/ml)
0	5.2×10^7
1	8.8×10^7
2	1.2×10^8
3	1.3×10^8
7	1.0×10^8



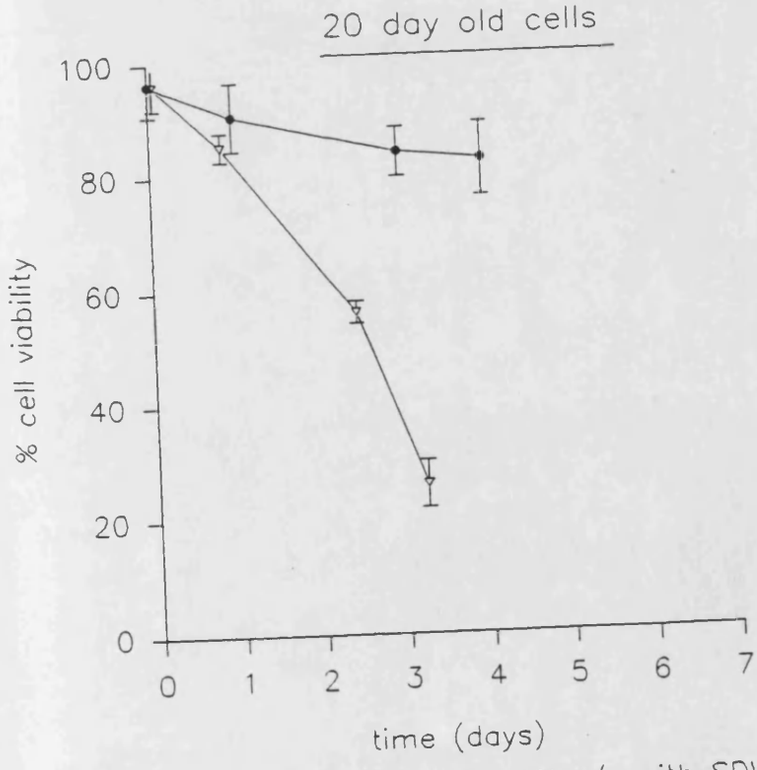
time (days)	bacterial no.s(cfu/ml)
0	7.4×10^7
1	2.4×10^7
3	3.4×10^7
4	6.5×10^8

Vertical bars represent SD.

Fig..3 . –% cell viability of cells from 10 and 20 day old suspension cultured cells of cultivar MCol 113 inoculated with Xcm isolate 2967



time (days)	bacterial no.s(cfu/ml)
0	4.0×10^7
1	5.3×10^7
2	1.5×10^8
3	1.6×10^8
5	1.3×10^8



time (days)	bacterial no.s(cfu/ml)
0	3.7×10^7
1	4.0×10^7
3	1.2×10^8

● – control plant cells 1% v/v with SDW

▿ treated plant cells 1% v/v with 1×10^7 cfu/ml of isolate 2967

Vertical bars represent SD.

5. Effect of bacterial cells contained in dialysis tubing on suspension cultured cells of cassava

'Dialysis cultures' of microorganisms have proved useful for coculture of plant cells with bacteria; bacteria are separated physically from plant cells but small molecules from host or pathogen which may function as inducers or as 'toxins' can be exchanged (Schultz & Gerhardt, 1969). This approach should establish if cell to cell contact is required for host cell killing and may assist in the identification of substances from plant-microoganism association involved in pathogenesis.

A 1% (v/v) suspension of 20 day old cassava cells of cultivar MCol 113 in 1/10th strength MS was inoculated with a suspension of Xcm isolate 2967 ($1x10^8$ cfu/ml suspended in 1/10th strength MS). Host-pathogen contact was prevented by placing the bacterial suspension in bags of dialysis membrane (VT31 size 8, molecular weight cut-off 12-14,000 Daltons, Medicell International Ltd.) sealed by knotting of the ends. Before use, the dialysis membranes were treated and sterilised in the way described in Maniatis et al. (1982) as untreated dialysis membrane proved to be toxic towards cassava cells. The controls consisted of plant cells suspended in the above medium at the above concentration and i) 5ml of the bacterial suspension added to give a final bacterial concentration of $1x10^7$ cfu/ml or ii) treated dialysis

bags containing 1/10th strength MS added into them. Every treatment was replicated 4 times. Samples were removed aseptically at time intervals and host cell viability was assessed by staining with FDA. Results presented in Table 12 show that the bacteria contained in the dialysis bags were unable to cause mortality of cassava cell upto 5 days in contrast to bacteria added directly to suspension cultured cells which reduced viability from approximately 75% to 11% in this period. Further observations could not be carried out as the cultures became contaminated with fungi; the contamination recurred in a repeated experiment.

Table 12- Effect of host-bacterial cell contact on host cell death

time (days)	% cell viability±SD		
	controls		treated
	1	2	
0	75.2±1.2	75.2±1.2	73.7±1.5
1	70.1±0.8	72.6±1.5	71.8±0.7
2	50.6±1.4	73.8±2.1	72.5±1.3
3	28.2±1.5	70.6±0.7	69.4±1.6
5	11.4±0.6	62.8±1.3	60.3±1.7

The inoculated flasks were incubated in a shaker at 25°C, 150rpm speed and 135uE $m^{-2}sec^{-1}$ light intensity

Control 1- flasks with 20 day old suspension cultured cells of cassava cultivar MCol 113 suspended in $1/10^{th}$ strength MS with Xcm isolate 2967 added at $1x10^{7}$ cfu/ml concentration

Control 2- flasks with 20 day old suspension cultured cells of cassava cultivar MCol 113 suspended in $1/10^{th}$ strength MS added with dialysis bags with 1/10 MS.

Treated- flasks with 20 day old suspension cultured cells of cassava cultivar MCol 113 suspended in $1/10^{th}$ strength MS with Xcm isolate 2967 added in dialysis bags at $1x10^{7}$ concentration.

DISCUSSION I

Various explants from cassava were cultured on a range of media to investigate the different morphogenic responses with the aim of obtaining a suitable *in vitro* system to study the interaction between isolates of *Xcm* and the host tissues.

The majority of treatments resulted in unorganised friable callus formation and some in adventitious root formation. In all 4 cassava cultivars tested, shoot formation could only be achieved by culturing stem nodes or shoot tips and in these cases the shoots were derived from existing meristems.

Kartha *et al.*, (1974) regenerated plants from the meristematic domes of 5 cassava cultivars and Kartha & Gamborg (1975) reported 90-95% regeneration of plants by the culture of apical meristems on MS medium supplemented with hormones. They have used these conditions to eliminate cassava mosaic virus from two Indian and Nigerian cultivars.

Stamp (1984) obtained callus proliferation and adventitious root initiation from a range of cassava explants on a wide range of treatments although the types of callus that developed varied in their compactness, wetness and colour. This was not observed in the present study as the callus that developed from all explant types

on all media was friable, unorganised and brownish white in colour.

None of the attempts in the present study yielded adventitious plant regeneration but there have been reports of success with stem callus (Tilquin, 1979) which could not be repeated by others; protoplasts (Shahin & Shepard, 1980), seed tissues (Stamp & Henshaw, 1986; Biggs et al, 1986) and immature leaf tissue (Stamp & Henshaw, 1986).

Regeneration of plantlets from stem nodes was achieved on semi-solid MS_{20} medium without any added hormones. This was carried out in a two stage procedure (i).stem node explants were cultured on MS_{20} and (ii). the individual nodes from the stems that developed were cultured on fresh MS_{20}. However, this process proved to be slow compared to the culture of stem nodes in liquid MS_{20} as shake cultures in the first stage which produced stems 2-5 times longer than those produced on the semi-solid medium. This could reflect the greater nutrient uptake when immersed in moving liquid medium.

Culturing stem nodes in liquid shake cultures has also been reported to be an efficient method in the propagation and in vitro tuberisation of potato (Rossel et al., 1987) and the method has also proved to be suitable for the in vitro propagation of various other

cultivars of cassava (N.J.Taylor, unpublished data).

The suitability of various tissue culture systems were investigated for their use in investigating pathogenicity of isolates and pathogenicity mechanisms of Xcm on cassava.

In this context, inoculation of callus tissues of various cassava cultivars with virulent isolates of Xcm appeared to be an unsuitable system. There was no increase over 48h in electrolyte leakage following inoculation. However, subsequent data on killing of suspension cultured cells, electrolyte leakage from bacteria infiltrated leaf discs and on appearance of symptoms in leaves suggest that longer periods of incubation should have been tried.

Callus tissues have been used as a system to determine genotypic expression of resistant and susceptible cultivars of various plant types. Helgeson et al. (1972, 1976) showed that calli from resistant tobacco varieties were colonised less rapidly and less extensively by Phytophthora parasitica var nicotiana than calli from a susceptible plant. Huang et al. (1989) found differential responses of callus tissues derived from resistant and susceptible clones of potato when inoculated with Pseudomonas solanacearum. The change in colour (green to brown) of potato callus due to infection by the pathogen has been used as an indication of

colonisation; but as none of the conditions tested in the present study produced green calli of cassava, change of colour of calli as an indication of bacterial growth could not have been used.

Frequently workers have failed to detect expression of resistance by callus derived from disease resistant plants. Holliday & Klarman (1979) did not find a correlation between resistance of soybean to Phytophthora megasperma in callus cutures and neither could Ingram (1969) show the susceptibility of different Brassica callus cultures to P. parasitica to be constant.

These findings and the lack of response of cassava callus cells to Xcm in this study could reflect the inherent autonomy of callus cells which especially in loosely packed unorganised friable callus, prevents collective participation thus preventing a coordinated response to any interaction.

Plantlets regenerated in vitro were also assessed for the possibility of using them to study the host-pathogen interaction. Some typical symptoms of bacterial blight could be reproduced by inoculating Xcm isolates to cassava plantlets in vitro. Although the first symptom i.e. wilting of the leaf at the point of inoculation was clearly apparent after 8-12 days, dependent on the virulence of the Xcm isolate, further assessment of the

degree of symptom production could not be carried out because the whole of the plantlet became chlorotic and wilted. This could have been because the inoculum level was too high in relation to the relative size of the host material so that the finer distinctions in symptom production could not be shown. Therefore, a range of lower inoculum levels should be tested to determine the optimum bacterial concentration that would show the gradual advancement of symptoms. The time required for total chlorosis and wilt of plantlets varied between isolates and was related to the slightly different levels of isolate virulence as found on cassava plants.

Others have used similar systems to evaluate pathogenicity of various bacteria. Work by Viseur & Tapia (1987) and Duron et al. (1987) to evaluate fire blight resistance in pears and apple has shown that in vitro plantlets could be used to establish a clear distinction between highly resistant and very susceptible cultivars although intermediate resistance was shown to be more difficult to assess. Rott & Chagvardieff (1987) developed a system of reproducing the symptoms of leaf scald disease of sugarcane by inoculating two strains of Xanthomonas albilineans to in vitro cultured plantlets.

A difference in symptom production between resistant and susceptible cultivars was not evident. However, to date no cassava lines with clear resistance to Xcm are

available to test in this system thus no conclusions as to the potential of the method can be drawn at this stage (Section II,2 & 3).

It can be concluded that like some other host-pathogen interactions tested in which plantlets have been used, the pathogenicity or virulence of Xcm isolates on cassava also could be monitored in this way as a fast, low cost screening method. It could especially be useful as a large scale screening method for testing bacterial mutants of altered pathogenicity.

Mass production of plantlets for such screening programmes could be achieved by the efficient method of culturing the stem node explants initially in liquid shake cultures.

Establishment of cassava cell suspension cultures from friable callus tissue allowed the development of another tissue culture system that responded in an apparently appropriate way to Xcm. Inoculation of suspension cultured cells of cassava with two virulent isolates of Xcm demonstrated the ability of the bacterium to affect the viability of cassava cells. The cassava cells became more susceptible with increasing age but cells of every age tested (eg.5,10 and 20days) showed a lag phase before loss of viability when the bacteria were added. It could be presumed that the bacterial action on the plant cells involves an initial biotrophic

phase before cell killing begins. Biotrophy is also apparent in delayed symptom production in whole plants infected with Xcm (Section II, 2) and in a number of other disease causing pseudomonads and xanthomonads and the delayed electrolyte leakage from leaf discs infiltrated with Xcm (Section II, 4). Roach and Garnett (1986) have also shown that suspension cultured cells of two cassava cultivars required about 72h for complete cell death when inoculated with two pathogenic strains of Erwinia herbicola.

There has been no previous report of the relationship between plant cell age and cell mortality when inoculated with a bacterium although this approach of coculture has often been used for many bacterial host interactions (Matthysse, 1987; Atkinson et al., 1985; Hsu & Goodman, 1978; Fett & Sacharius, 1982; 1983; Roach & Garnett, 1986). The conclusions drawn by these workers could have been different had they used a similar approach to the one described here.

The possible mechanisms of cell killing by Xcm are investigated later (Section III).

1/10th strength MS was identified as a suitable medium to suspend bacterial and host cells, as it maintained the viability of both bacterial and plant cells without allowing prolific bacterial growth.

Extensive bacterial growth may result in the death or reduced growth of the plant cells due to limitation of nutrients or oxygen, alteration of the constituents or pH of the medium. 1/10th MS medium was also reported to be suitable to suspend carrot and tobacco cells to observe the elicitation of an HR like reaction when inoculated with the incompatible bacterium, Pseudomonas syringae pv phaseolicola (Matthysse, 1987). It was considered to be similar to the dilute nutrient conditions which would be found in the intercellular spaces of leaves.

Although 1/10th strength MS allowed bacterial cell survival, the bacterial numbers did not increase in this medium unless added with the plant cells. This indicates that , as happens in vivo, the bacterial cells presumably obtain carbon and additional required nutrients from cassava cells; this may be achieved by controlled induction of host cell leakage but eventual killing of host cells coincided with maximal growth rate of Xcm. Additional carbon may be derived from host cell wall polysaccharide degradation (see Section III,B).

As an alternative approach Fett & Sacharius (1983) in an experiment to determine phytoalexin elicitation in soybean cell suspension cultures inoculated with P. syringae substituted maltose, which could not be utilised by P. glycinea or P. phaseolicola strains, for sucrose in the medium so that the pathogen had to interact with

the host cells in order to gain the required carbon for its growth.

The increase in bacterial numbers when cocultured with cassava cells also could be attributed to a stimulus produced by cassava cells in the suspension culture. A growth stimulus was reported to be produced by suspension cultured cells of a susceptible potato cultivar in response to inoculation with P. infestans. (Ingram & Robertson, 1965).

Comparison of the 3 types of bioassays reveals the cassava cell suspensions as the most suitable for studying the host-pathogen interaction. It can be performed over a short time period, reducing likelihood of false results arising due to contamination of the test with spurious microorganisms. The host material is also of a more uniform nature allowing better reproducibility of the experiments. One drawback of the viability assay is that it is lengthy and subjective. Automation could be achieved by detection of fluorescein in viable cells with a fluorimeter, but criteria such as uneven cell distribution and background fluorescence of the medium should be considered. Extraction of fluorescein from cells should overcome these limitations but this also can be a lengthy process. It should also be noted that the uniformity of the cell population which allows for timed sampling and uniform host cell responses also precludes

the observation of responses of differentiated cell types to the pathogen and of the interactions and communications among various plant cell types. Suspension cultured cassava cells are rapidly growing and dividing, are non chlorophyllous and exist in an aqueous environment. The extracellular environment of cultured cassava cells may provide more readily available water, nutrients and minerals for bacterial growth than does the interior of a plant. These differences should be considered when interpreting results obtained with this in vitro system.

This system could also be used for the selection of resistant plants if an efficient regeneration procedure from suspension cultures was available as it would be a suitable method for screening a large number of individuals at the same time. Methods for efficient regeneration from suspension cultured cassava cells are being investigated (Taylor, unpublished data).

The effect of culture filtrates of the bacterium could also be tested and used as a selection pressure for resistant lines. A highly embryonic cell suspension of alfalfa derived from a genotype sensitive to Fusarium oxysporum has been successfully used for selection in vitro for resistance to culture filtrates of Fusarium oxysporum, F. solani and F. avenaceum (Binarova et al., 1990).

When the bacteria were contained in a dialysis bag and added to plant cells, they were unable to cause death of cassava cells. This suggests that either the bacterial factor that causes cell killing is of high molecular weight and cannot permeate through the pores in the dialysis membrane and/ or that contact between the plant and bacterial cells was essential for the process to be initiated. Hignett et al. (1983) established that host bacterial cell contact is a necessary prerequisite for the induction of host (pear) eletrolyte leakage when inoculated with the pathogen Erwinia amylovora. This was confirmed by Youle & Cooper (1987) who showed that the coincubation of host (apple) and Erwinia amylovora cells in suspension separated by dialysis tubing did not cause host cell death.

Correlation between host cell killing by pathogenic isolates but not by non pathogenic mutants of Xcm would be additional information on the validity of the test. In the fire blight study described above, avirulent mutants (other than EPS$^-$) of E. amylovora fail to kill host cells (Youle & Cooper, 1987; Cooper, unpublished data).

Similarly, expression of disease resistance at this stage would be of considerable interest and value. Although callus initiation and growth from resistant cultivars was achieved, it was not possible to initiate and maintain suspension cultures under the same

conditions as for the susceptible cultivars thus comparisons could not be made. Suspension cultures and calli have been used to demonstrate the difference in reactions shown by susceptible and resistant hosts. Helgeson et al. (1972; 1976) showed that tissue cultures of resistant tobacco cultivars inoculated with Phytophthora parasitica var nicotianae were colonised less rapidly and less extensively than tissue cultures of susceptible cultivars. Hypersensitivity to incompatible pathogens has also been demonstrated using with tissue culture systems (Atkinson et al., 1985; Matthysse, 1987; Huang et al., 1989).

Coculture of Xcm and cassava suspension cultured cells could also be a suitable system for extraction of the bacterial products at stages during the interaction The bacterial products, if extracellular, could be extracted relatively easily with little interference from intracellular plant products in contrast to inevitable contamination by extraction from plant tissues. If the pathogenicity factor is host induced as in the sugarcane pathogen Helminthosporium sacchari which requires the host leaf exudate serinol for the induction of HS toxin (Pinkerton & Strobel, 1976), or as in some cell wall-degrading enzymes of pathogens which require mono or dimeric degradation products from cell wall polysaccharides (Cooper, 1983), this system should allow

the production and expression of such a factor. It follows that host induced pathogenicity factors may not be detected in routine *in vitro* culture of microbial pathogens.

In conclusion, it can be stated that plantlets developed *in vitro* and suspension cultured cells of cassava could be used to monitor the activity of *Xcm* isolates on cassava. This last system later also proved to be a suitable assay for a putative toxin produced by of *Xcm*.

SECTION II- PATHOGENICITY OF Xcm ISOLATES ON SUSCEPTIBLE AND RESISTANT CASSAVA VARIETIES

Preliminary studies on Xcm isolates were carried out with a view of determining their nutrient requirements, growth characteristics, and sensitivity to antibiotics.

Pathogenicity of Xcm isolates from South America and Africa were tested against cassava cultivars of known resistance/susceptibility. The aims were to (i).identify the best methods of inoculation (ii).assess the patterns of symptom development and the differences in time required for disease development and (iii).the effects of different levels of inoculum on disease development.

1. Preliminary studies on bacterial characteristics

Preliminary investigations on bacterial isolates were conducted to determine their growth rate and growth requirements.

a).Measurement of bacterial growth

(i).Growth in complete NYGB (Turner et al., 1984) medium

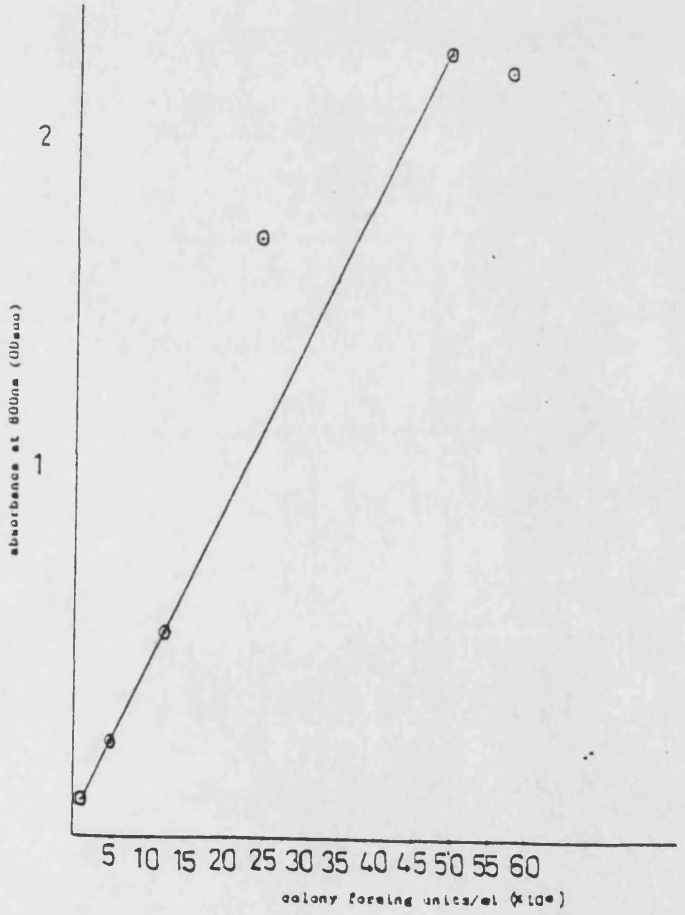
Growth in complete NYGB medium was studied as described in Materials and Methods, 6c.

Fig. 4 shows the standard curve of absorbance at

600nm versus the number of colony forming units per ml (cfu/ml) of isolate 3194 obtained by serial dilutions of bacterial suspensions of known absorbance (measured at 600nm) made in SDW and plated in triplicate in NYGA.

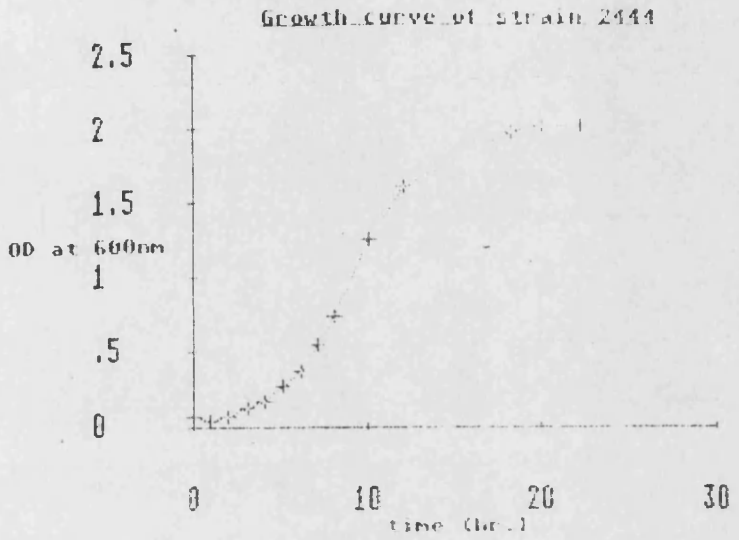
The change in absorbance at 600nm of 8 strains of Xcm grown in NYGB was observed at hourly intervals over a 25h period. All bacterial strains had a lag phase of about 3h, a linear phase of about 12- 15h and they reached stationary phase by about 18h (Fig. 5I-5VIII).

Fig. 4- Standard curve of Xcm isolate 3194.

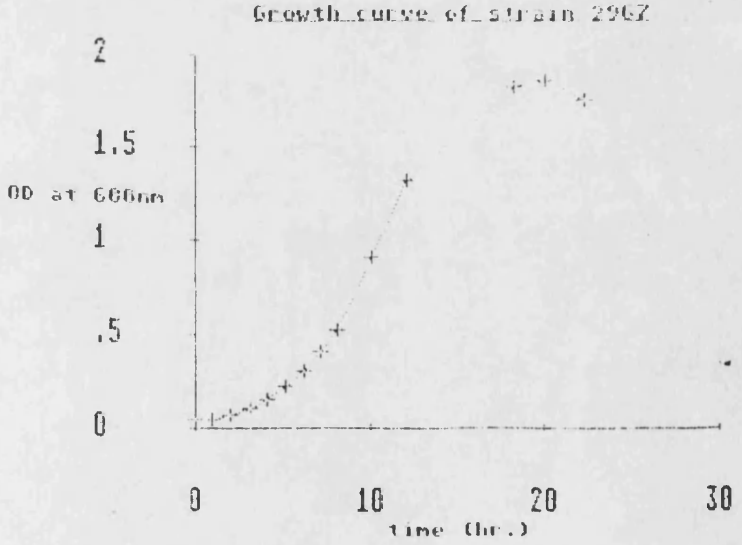


All bacterial growth for further experiments was carried out under similar culture conditions for 12h.

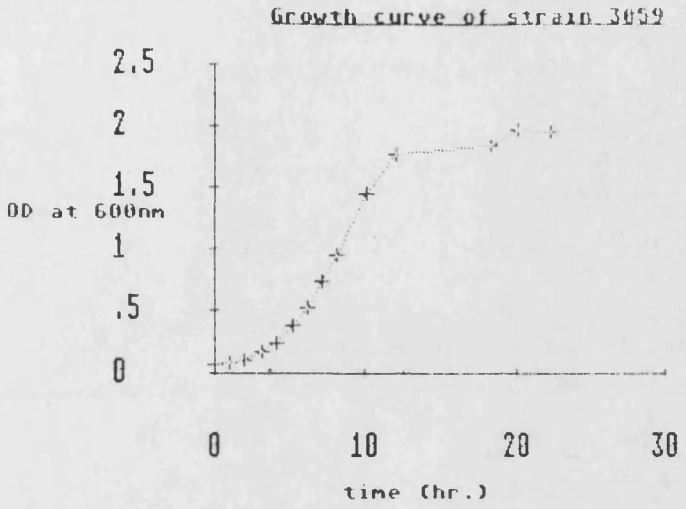
Fig.5I-5VIII- Growth curves of the Xcm isolates (absorbance at 600nm versus time)



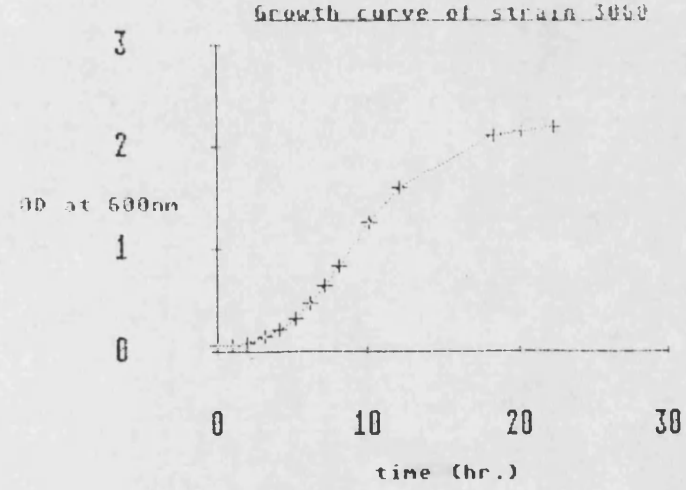
II



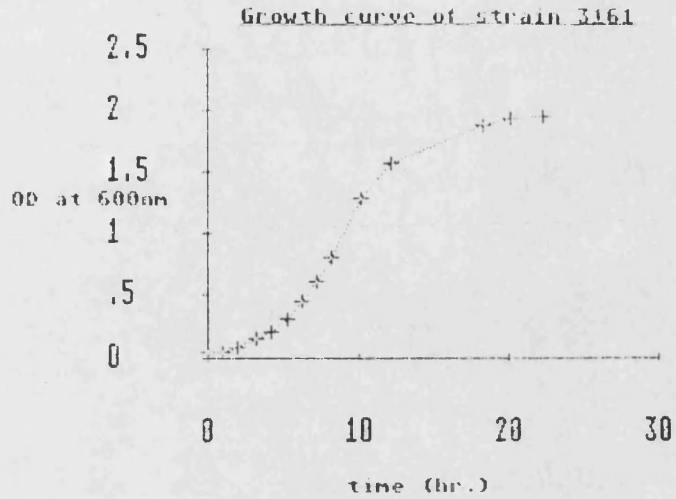
III


Growth curve of strain 3059
2.5
2
1.5
OD at 600nm
1
.5
0
0
10
20
30
time (hr.)

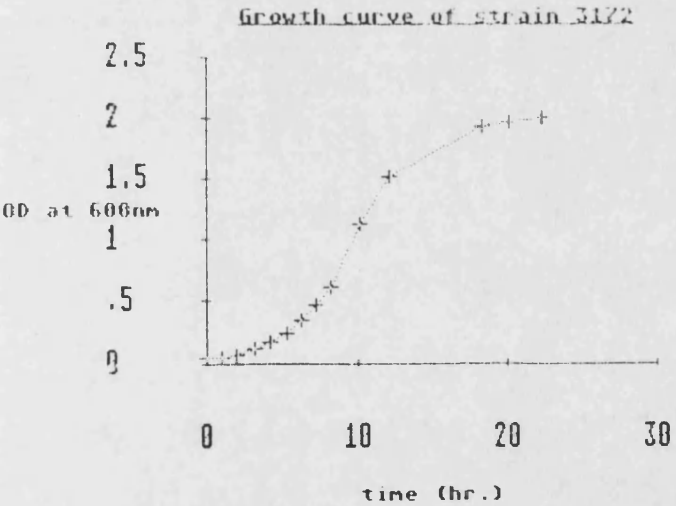
IV


Growth curve of strain 3060
3
2
OD at 600nm
1
0
0
10
20
30
time (hr.)

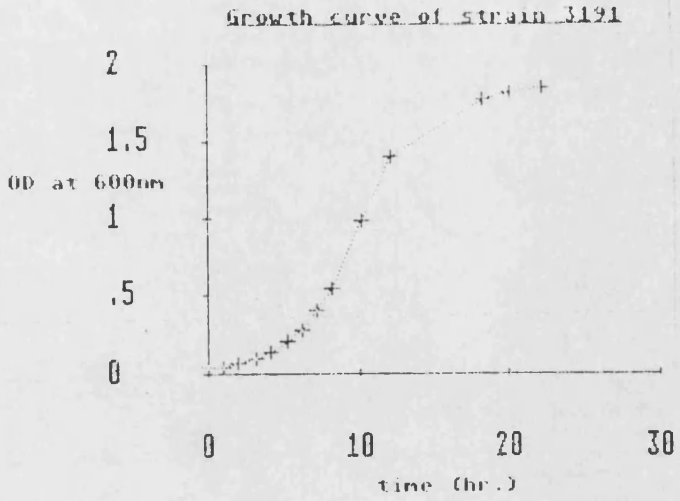
V


Growth curve of strain 3161
2.5
2
1.5
OD at 600nm
1
.5
0
0
10
20
30
time (hr.)

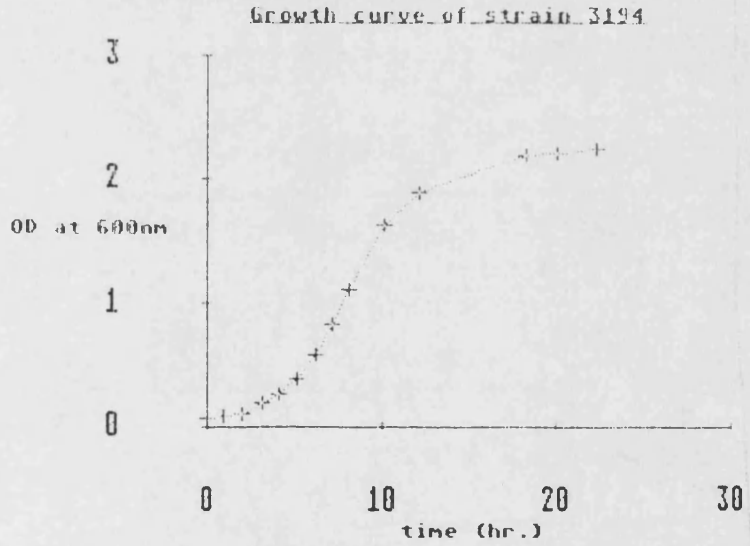
VI


Growth curve of strain 3172
2.5
2
1.5
OD at 600nm
1
.5
0
0
10
20
30
time (hr.)

VII



VIII



All 8 Xcm isolates were grown overnight in NYGB at 30°C and 1ml from each culture was added to 100ml of fresh NYGB in 250ml flasks. The flasks were then incubated at 30°C in a gyratory shaker (150 rpm) and OD measurements at 600nm obtained at hourly intervals using a PU 8650 visible spectrophotometer. There were 4 replicate flasks for each isolate.

(ii). Growth of bacteria in minimal media

The growth of all 8 isolates of Xcm was tested in minimal medium A and minimal medium B (see Appendix 1 for details).

Single colonies from bacterial isolates grown on NYGA plates for 2 days were streaked onto plates of Minimal medium A and B separately and incubated for 4 days at 30°C and observed for bacterial growth. There were 4 replicate plates for each isolate in each medium.

None of the Xcm isolates grew well in either of the media, indicating that they had an additional nutrient requirement.

The growth was also tested in minimal medium B supplemented with either 10 µg/ml methionine, 1 µg/ml nicotinic acid or 10 ug/ml glutamic acid. These plates were inoculated, incubated and replicated as stated above.

These isolates varied in their nutrient requirements (Table 13). Isolates 3194, 2444 and 3059 were able to utilise any of these 3 amino acids while isolates 2967, 3060, 3161, 3191 and 3172 showed auxotrophy for methionine. Consequently, Mm B with added methionine was used in further experiments.

Table 13 -Testing for bacterial auxotrophy

isolate	mm A	mm B	mm B with meth.	mm B with nic. acid	mm B with glu. acid
2444	˜	˜	+	+	+
2967	˜	-	+	-	-
3060	-	˜	+	-	-
3059	˜	˜	+	+	+
3161	˜	-	+	-	-
3172	˜	˜	+	-	-
3191	-	-	+	-	-
3194	-	-	+	+	+

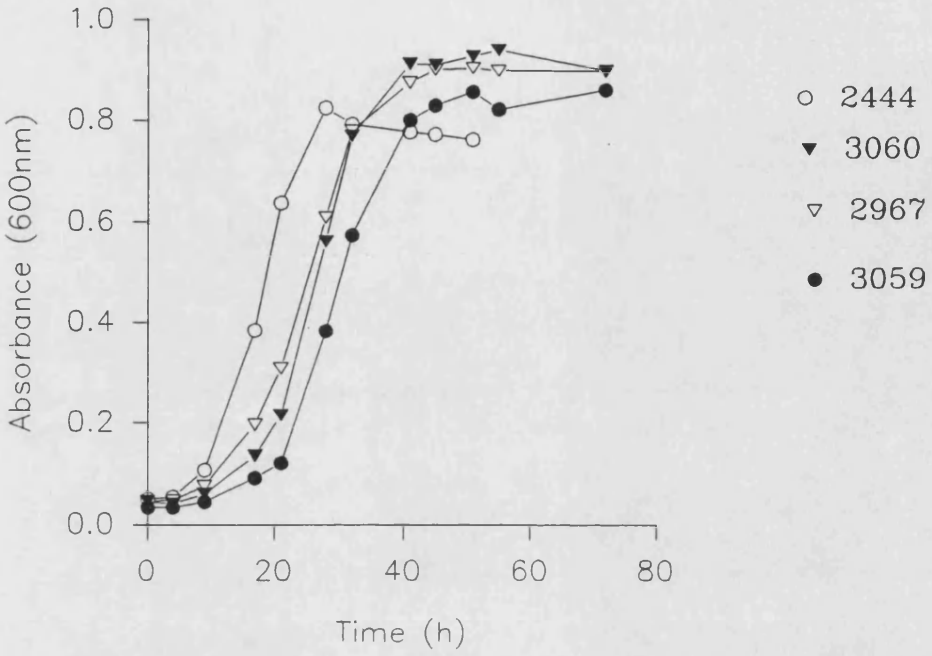
mm A -minimal medium A mm B- minimal medium B
meth. - methionine (10 ug/ml)
nic. acid-nicotinic acid (1ug/ml)
glu. acid- glutamic acid (10 ug/ml)

bacterial growth:- - none ˜ weak growth + normal growth
There were six replicate plates for each treatment.

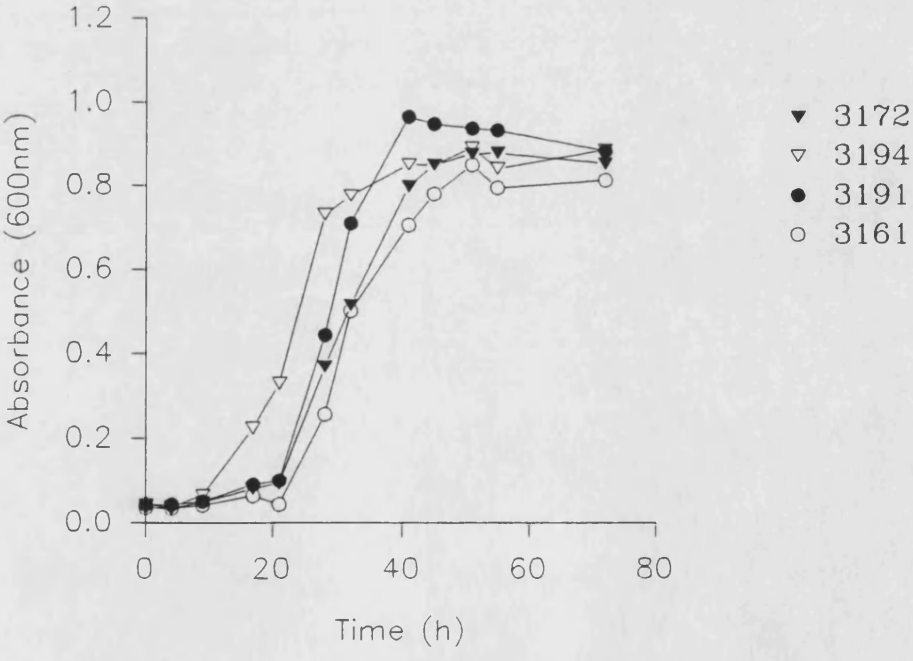
Growth of all 8 Xcm isolates in Mm B broth supplemented with 10ug/ml methionine was tested over a 72h period.

The cultures reached exponential phase after ca. 10h and stationary phase after ca. 40-48h (Fig. 6).

Fig. 6 Growth of Xcm isolates in minimal medium supplemented with 10ug/ml methionine



SD varied from 0.02–0.13



SD varied from 0.01–0.23

Each point on both graphs is a mean value of four replicates.

b).Bacterial resistance to antibiotic treatment

The purpose of this experiment was to examine the sensitivity of Xcm to various antibiotics with the aim of identifying suitable antibiotics which could be used in selective media for reisolation.

Concentrated stock solutions of antibiotics to be used were made up as follows-

Chloramphenicol (Sigma)- 50 mg/ml of the sulphate in 50% ethanol sterilised by filtration

Kanamycin (Sigma) - 25 mg/ml of the sulphate in SDW

Streptomycin (Sigma) - 100 mg/ml of the sulphate in SDW

Ampicillin (Sigma) - 25 mg/ml in SDW

Bacterial growth was observed on separate NYGA plates with 5, 10, 20ug/ml Chloramphenicol, 10, 25, 50ug/ml Kanamycin, 50, 100, 250 ug/ml Streptomycin and 10, 25, 50ug/ml Ampicillin.

Bacteria were streaked onto NYGA plates containing antibiotics at the above concentrations, incubated at 30°C and observed after 3 days for growth (Table 14).

Table 14 - Bacterial resistance to antibiotics

bacterial isolate	antibiotic and concentration (ug/ml)											
	ampicillin			chloram.			streptomycin			kanamycin		
	10	25	50	5	10	20	50	100	250	10	25	50
2444	+	+	+	˜	˜	-	-	-	-	˜	-	-
2967	+	+	+	˜	-	-	˜	-	-	˜	˜	-
3060	+	+	+	˜	-	-	-	-	-	˜	-	-
3059	+	+	+	˜	-	-	˜	-	-	˜	˜	-
3161	+	+	+	˜	-	-	˜	-	-	˜	˜	-
3172	+	+	+	˜	˜	-	-	-	-	˜	-	-
3191	+	+	+	˜	-	-	-	-	-	˜	˜	-
3194	+	+	+	˜	-	-	˜	-	-	˜	˜	-

+ growth ˜ weak growth - no growth
There were six replicate plates for each treatment.

Of the antibiotics tested, chloramphenicol proved to be the most effective, having bactericidal activity at concentrations as low as 20ug/ml. Streptomycin and kanamycin controlled bacterial growth at 100 ug/ml and 50 ug/ml respectively whereas ampicillin did not inhibit growth at any of the concentrations tested.

2. Pathogenicity of Xcm isolates

In order to observe symptom development and to test the most appropriate inoculation method, a series of experiments was conducted using cassava cultivars of

known resistance and susceptibility inoculated in various ways.

(i). Symptom development on susceptible cassava cultivar MCol 22 inoculated with Xcm isolate 3194

Two month-old plants of cassava cultivar MCol 22 were inoculated with Xcm isolate 3194 using several inoculation procedures (Materials and Methods, 9), following inoculation, the plants were maintained in a Saxcil growth chamber at 30°C daytime and 25°C night time temperatures for 22 days and observed for symptom development. Six replicate plants were inoculated for each treatment.

Symptoms ranged from localised lesions on leaves sprayed with bacteria to systemic wilting and stem tip die back after 16 days with the stem stabbing and stem injection methods (Table 15, Plate 5). Injection of bacterial suspension to the base of the stem produced stem discoloration but no systemic wilting and injection of bacterial suspension into the leaf petiole caused leaf abscission. Thus stabbing the stem at 3rd and 4th leaf axils with a needle dipped in the bacterial suspension and injecting the stem at 3rd and 4th leaf axils with 0.5ml of the bacterial suspension was used in further experiments.

Table 15 -Symptom development of plants of cultivar MCol 22 inoculated with Xcm isolate 3194 using different methods

method of inoc.ⁿ	no. of days after inoc.ⁿ	symptoms
1).spraying with the bacterial suspension ($1x10^8$ cfu/ml)	7	appearance of localised lesions on sprayed leaves. These did not spread to the leaves that developed later.
2).stabbing the stem at 3rd and 4th leaf axils with a sterile needle dipped in the bacterial suspension	10	one leaf wilted at point of inoculation
	14	more leaves wilted above point of inoculation
	20	tip die-back of stem
3).injecting 1 ml of bacterial suspension ($1x10^8$ cfu/ml)with a syringe to-		
a].stem at 3rd and 4th leaf axils	10	one leaf wilted at the point of inoculation
	14-15	more leaves wilted above point of inoculation
	18	tip die-back of stem
	20	leaves wilted below point of inoculation. stem discolouration.
b].base of stem	21	stem discoloured and one leaf wilted at point of inoculation.
c].base of petiole	12	leaf wilted and dropped no further spread of disease.
d].tip of petiole	13	same as above.

(ii).Further studies of the pathogenicity of Xcm isolates

The pathogenicity of some of the Xcm isolates was further examined on cassava cultivars which were rated as either susceptible or resistant (Lozano & Laberry, 1982; CIAT, 1975). Various methods of inoculation were examined again.

Suspensions of Xcm isolates 2444, 3172, 3194, 2967 and CIAT 1222 in SDW ($1x10^8$ cfu/ml) were inoculated separately into 2 month old cassava plants of cultivars MCol 22 (susceptible), MCol 113 (susceptible), MNGA 1 (resistant) and MVen 77 (resistant) using the following methods of inoculation.

a).injecting 0.5ml of the bacterial suspension into the cassava stem at the fourth leaf axil.

b).infiltration of 0.5ml of the bacterial suspension to the abaxial surface of the middle lobe of the third and fourth leaves.

c).clipping the mid lobe in half of the third and fourth leaves of cassava plants with scissors dipped in the bacterial suspension.

a). The effect of injecting the host stem with different Xcm isolates

The pathogenicity of Xcm isolates 2444, 2967, 3172 and 3194 was evaluated on cultivars MNGA 1, MCol 22 and MCol 113 using the stem injection technique.. A disease index was used to evaluate the development of symptoms over time.

Table 16 - Effect of stem injection of 4 Xcm isolates towards cassava cultivars MNGA 1, MCol 22 and MCol 113

cultivar	no. of days after inoculation	symptom development induced by isolates 2444	2967	3172	3194
		disease rating			
MCol 22	10	0	1	0	1
	12	0	1	1	1
	14	0	2.0	2.0	2.0
	16	1	2.0	2.0	2.0
	18	1	3.0	3.0	3.0
	20	1	3.5	3.5	3.5
	24	1	5.0	5.0	4.0
MNGA 1	10	0	0	0	0
	12	0	1	1	0
	14	0	1	1	1
	16	0	1.5	1.5	1.5
	18	1	2.0	2.0	2.0
	20	1	3.0	3.0	2.5
	24	1	3.5	3.5	3.5
	30	1	5.0	4.0	4.0
MCol 113	10	0	1	1	1
	12	0	1.0	1.0	1.0
	14	0	2.0	2.0	2.0
	16	1	2.0	2.0	2.0
	18	1	3.0	3.0	3.0
	20	1	3.5	3.5	3.5
	24	1	5.0	5.0	4.0

The disease index used-

0- no symptom

1- wilting and shrivelling of one or a restricted number of leaves near the point of inoculation

2- wilting and shrivelling of leaves above the point of inoc.ⁿ

3- tip die-back

4- additional leaf wilting below the point of inoculation and stem discolouration

5- wilt of the whole plant

The control plants were treated similarly with SDW. There were six replicates for each treatment for each cultivar.

Plants of cultivar MCol 22 inoculated with Xcm isolates 3172, 2967 and 3194 showed wilting and shrivelling of leaves near the point of inoculation within 10 days of inoculation (Table 16). Wilting of leaves above the points of inoculation occurred within the next 2 days and tip dieback was apparent after 18 days. Isolates 2967 and 3172 also induced wilting of the whole plant while isolate 3194 did not and maybe considered as slightly less virulent than isolates 3172 and 2967. However, even by the end of the experiment (30 days) isolate 2444 had only caused wilting at the inoculation points and thus can be considered as the least virulent of the isolates tested. (Plate 6).

Symptom development in plants of cultivar MCol 113 followed a similar pattern and again it was apparent that isolates 2967 and 3172 were more virulent than 3194. Isolate 2444 was the least virulent on MCol 113 as was on MCol 22.

Cultivar MNGA 1 responded similarly to bacterial infection except that there was a delay in the development of each symptom.

Plate 5- Two-month-old plants of susceptible cassava cultivar MCol 22 inoculated by stem stab method (Materials & Methods -) with Xcm isolate 3194, 25 days after inoculation.

Plate 6- Two-month-old plants of susceptible cassava cultivar MCol 22, 25 days after inoculation by stem stab method with A)SDW (control) suspensions of Xcm isolates B)2444 C)3194 D)2967 and E)3172.

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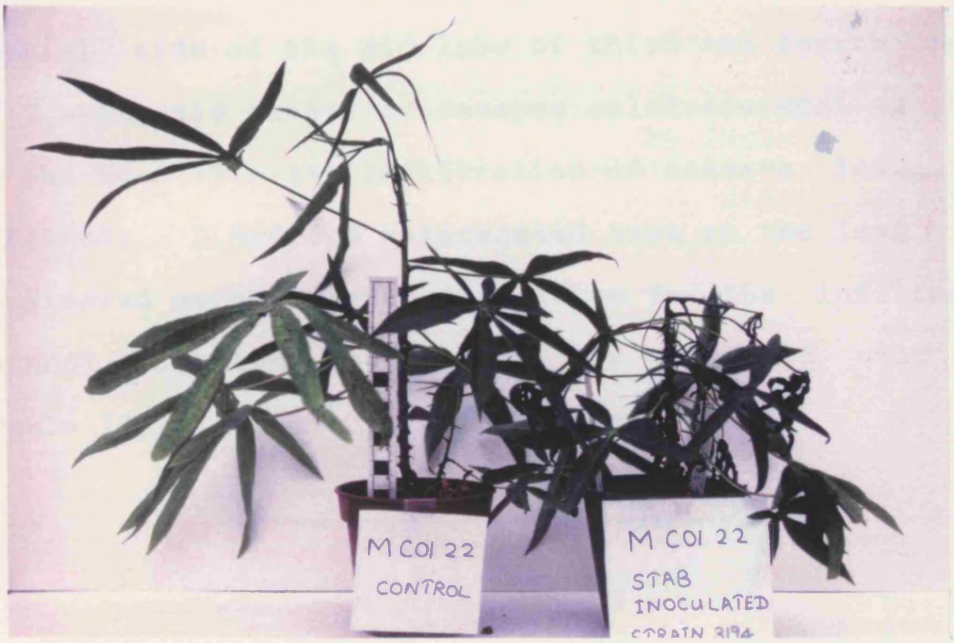

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b).The effect of infiltrating cassava leaves with Xcm

0.5ml of Xcm isolates 3194, 2967, 3172 and CIAT 1222 suspended in SDW ($1x10^8$ cfu/ml) were infiltrated into the abaxial side of the mid lobe of third and fourth leaves of 2 month old plants of cassava cultivars MCol 22, MNGA 1 and MVen 77. (As infiltration of cassava leaves was difficult, 1.5x0.5cm watersoaked area on the leaf were considered as the standard inoculum for the infiltration method). Symptom development was observed over time (Table 17, Plates 7, 8, 9 and 10).

Table 17 - Effect of leaf infiltration of Xcm isolates 3194, 2967, 3172 and CIAT 1222 on cassava cultivars MCol 22, MNGA 1 and MVen 77 when infiltrated into leaves.

cultivar	no. of days after inoculation	symptom development induced by isolates			
		3194	2967	3172	1222
		disease index			
MCol 22	3	1	1	1	1
	6	2	2	2	2
	8-9	3	3	3	3
	10-12	4	4	4	4
	15-16	5	5	5	5
	20	5	5	5	5
MNGA 1	3	1	1	1	1
	6	2	2	2	2
	8-9	2	2	2	2
	10-12	3	3	3	3
	14	4	4	4	4
	18	5	5	5	5
	22-24	5	5	5	5
MVen 77	3	1	1	1	1
	6	1	2	2	1
	8-9	2	2	2	2
	10-12	3	3	3	2
	14	4	4	4	3
	18	5	5	5	5
	22-24	5	5	5	5

Disease Index -
0- no symptoms
1- >0 ≤20% of the infected lobe affected
2- >20 ≤40% of the infected lobe affected
3- >40% ≤60% of the infected lobe affected
4- >60% ≤80% of the infected lobe affected
5- >80%-100% of the whole leaf affected

When 0-20% of the infected lobe was affected, (after 3 days) the infiltrated area on the leaf had necrosed (1)

and the necrosis spread to a localised area (20-40% of leaf area) around the infiltration (2) after 6 days. 40-60% of the infiltrated lobe wilted above the area of infiltration after about 9 days (3) which developed into wilting of the whole lobe (4). The entire leaf wilted after about 16 days (5).

Symptom development on MNGA 1 and MVen 77 leaves followed a similar pattern although there was an approx. 2 days delay than in MCol 22.

Plate 7- Leaf lobe of susceptible cassava cultivar MCol 22 3 days after inoculation with Xcm isolate 2967. Inoculation was conducted by infiltrating the leaf 0.5ml of bacterial suspension ($1x10^8$ cfu/ml).

Plates 8-10- Inoculation of leaves with Xcm isolate 2967 by infiltration.

Plate 8- MVen 77 inoculated with $1x10^7$ cfu/ml after 8 days.

7

8

Plate 9- MCol 22 inoculated with $1x10^8$ cfu/ml suspension after 15 days.

Plate 10- MNGA 1 inoculated with $1x10^8$ cfu/ml suspension after 15 days.

9

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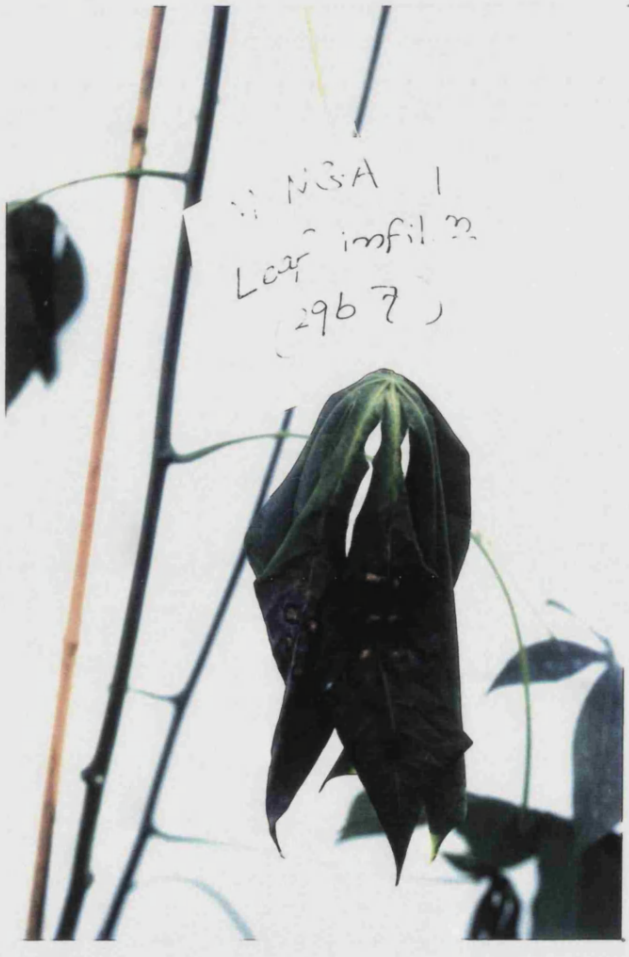

c).The effect of Xcm isolates when inoculated using the leaf clip method

In this experiment, the plants were inoculated using the leaf clip method of CIAT (1975). Hence, the middle lobe of the third and fourth leaves of cassava cultivars MCol 22, MNGA 1 and MVen 77 were clipped in half using flame sterilised scissors dipped in suspensions of Xcm isolates CIAT 1222, 3194, 2967 and 3194 ($1x10^8$ cfu/ml). Symptom development was observed at time intervals (Table 18).

There was no difference between the four Xcm isolates in causing symptom production. By six days all four isolates had caused the cut edges of leaves to necrose (0.5). The necrosis spread back gradually from the cut edge (Plate 11)(1,2,3,4) ultimately causing the whole leaf to wilt and drop off by approx. 17 days (5). Symptom development was initially slower on cultivars MNGA 1 and MVen 77 but followed a similar pattern as that on MCol 22.

Table 18 - Effect of Xcm isolates 3194, 2967, 3172 and CIAT 1222 on cassava cultivars MCol 22, MNGA 1 and MVen 77 when inoculated using the leaf clip method.

cultivar	no. of days after inoculation	isolate 3194	2967	3172	1222
		symptom development			
MCol 22	6	0.5	0.5	0.5	0.5
	9	1	1	1	1
	13-15	3	3	3	3
	17	5	5	5	5
	20-22	5	5	5	5
MNGA 1	6	0.5	0.5	0.5	0.5
	9	1	1	1	1
	13-15	2	2	2	2
	17	5	5	5	5
	20-22	5	5	5	5
MVen 77	6	0.5	0.5	0.5	0.5
	9	1	1	1	1
	13-15	2	2	2	2
	17	5	5	5	5
	20-22	5	5	5	5

Disease Index - 0 no symptoms
1- >0 ≤20% of the infected lobe affected
2- >20 ≤40% of the infected lobe affected
3- >40% ≤60% of the infected lobe affected
4- >60% ≤80% of the infected lobe affected
5- >80%-100% of the whole leaf affected

Both methods of leaf inoculation resulted in symptoms of a similar nature i.e. necrosis around the area of inoculation and subsequent wilt of the leaf. In each instance, when the whole leaf wilted, the petiole also discolured and wilted and this resulted in leaf abscission. There was no external indication of symptoms becoming systemic.

Plate 11- Leaves of cassava cultivars MCol 22 (susceptible) and MNGA 1 (resistant) 12 days after inoculation with Xcm isolate 2967.
Inoculation was by clipping the mid lobe of third or fourth leaf with flame sterilized scissors dipped in a suspension of $1x10^9$ cfu/ml.

Plate 12- Colonies of Xcm isolate 2967 reisolated from inoculated stem of susceptible cassva cultivar MCol 22. Note the typical cream coloration and muciod appearance.

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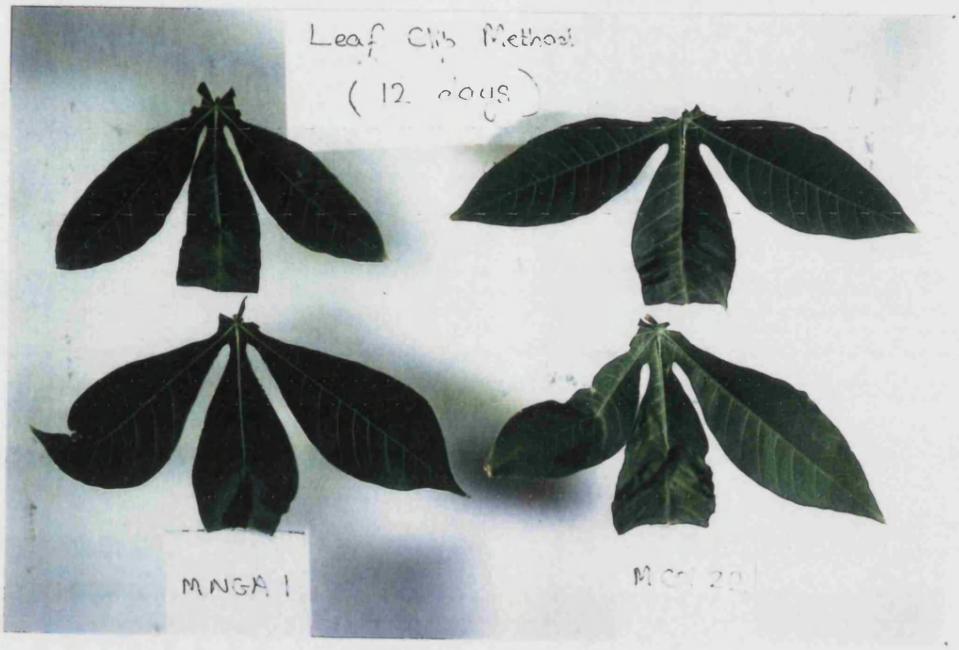

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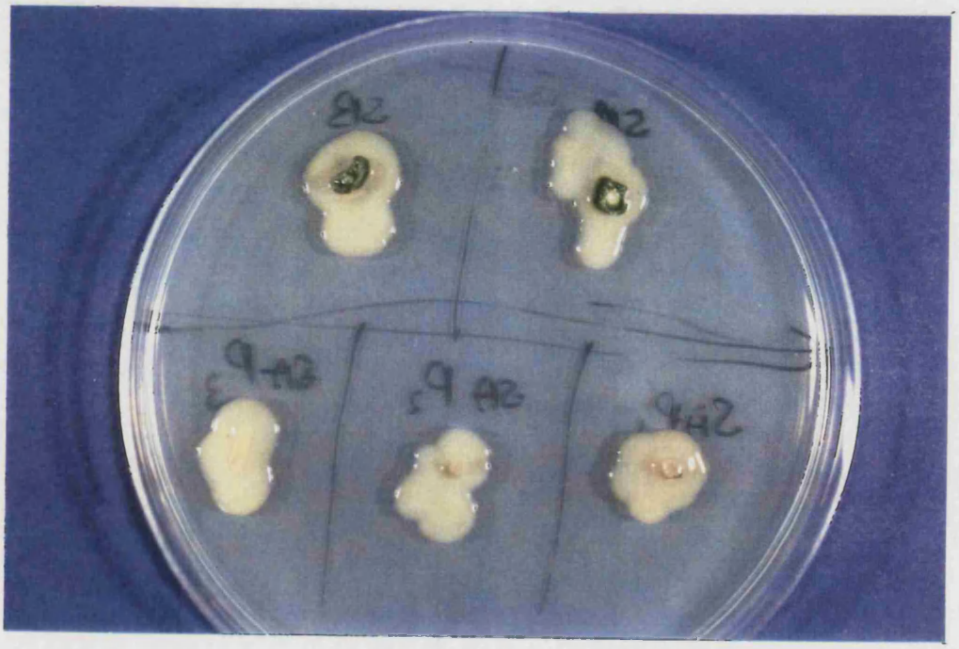


c).**Effect of different inoculum levels on disease development**

The effect of different levels of inocula on disease development was then studied using an isolate shown to be virulent in previous experiments. Hence, a suspension of Xcm isolate 2967 in SDW was prepared (Materials and Methods, 7) to give a concentration of $1x10^8$ cfu/ml. This concentration was diluted with SDW to give 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 cfu/ml concentrations. 0.5ml of each suspension was introduced into the abaxial surface of the mid lobe of the fourth leaf of 2 month old plants of cassava cultivars MCol 113, MNGA 1 and MVen 77 separately (this resulted in a watersoaked area of about 1.5x0.5cm) (Table 19).

Inoculum concentrations 10^8-10^5 caused a similar symptom progression in cultivar MCol 113 which completed the disease cycle ie. caused the inoculated leaf to abscise after 20 days (Table 19). Concentrations as low as 10^3 had also caused the whole of the infiltrated lobe to wilt after 12 days and after 20 days the the leaf had abcissed. In cultivar MCol 113 all concentrations of the Xcm had caused the whole of the inoculated cassava leaf to wilt after 15 days and after 20 days all inoculated leaves had dropped off.

Table 19- The effect of different concentrations of Xcm isolate 2967 into leaves of cassava cultivars MCol 113, MNGA1 and MVen 77

cultivar	day	symptoms induced by different conc.s							
		10^8	10^7	10^6	10^5	10^4	10^3	10^2	10
		disease index							
MCol 113	4	1	1	1	1	0	0	0	0
MNGA 1		1	1	1	1	0	0	0	0
MVen 77		1	1	1	1	0	0	0	0
MCol 113	6	2	2	2	2	1	1	0	0
MNGA 1		1	1	1	1	0	0	0	0
MVen 77		1	1	1	1	0	0	0	0
MCol 113	8	3	3	3	3	2	2	0	0
MNGA 1		2	2	2	2	1	1	0	0
MVen 77		2	2	2	2	1	1	0	0
MCol 113	12	4	4	4	4	4	4	1	0
MNGA 1		3	3	2	2	2	2	0	0
MVen 77		3	3	2	2	2	2	0	0
MCol 113	15	5	5	5	5	5	5	2	2
MNGA 1		4	4	4	4	4	4	1	1
MVen 77		4	4	4	4	4	4	1	1
MCol 113	18	5	5	5	5	5	5	5	5
MNGA 1		5	5	5	5	5	5	1	1
MVen 77		5	5	5	5	5	5	1	1
MCol 113	20	5	5	5	5	5	5	5	5
MNGA 1		5	5	5	5	5	5	1	1
Mven 77		5	5	5	5	5	5	1	1

Disease Index -

0- no symptoms
1- >0 ≤20% of the infected lobe affected
2- >20 ≤40% of the infected lobe affected
3- >40% ≤60% of the infected lobe affected
4- >60% ≤80% of the infected lobe affected
5- >80%-100% of the leaf affected

There were six replicate leaves for each concentration for each cultivar.

The pattern of symptom development in cultivars MNGA 1 and MVen 77 was similar to that of Mcol 113 in that the higher concentrations tended to produce more symptoms but once again there was a slight delay of a few days for symptoms to become apparent in these cultivars compared to the susceptible MCol 113. Also, unlike in cultivar MCol 113, the inoculum concentrations 10^2 and 10 failed to cause more damage than a slight yellow discolouration in the infiltrated leaves of cultivars MNGA 1 and MVen 77.

3.Movement and persistance of Xcm in leaves, petioles and stems of cassava

The outcome of infection may depend upon the ability of the pathogen to multiply rapidly within host tissues, outstrip or resist the deployment of host defence mechanisms and to produce pathogenicity factors to facilitate movement in the host plant. The bacterial movement in artificially infected plants was initially studied over a time period qualitatively using reisolation to determine bacterial spread.

(i).Qualitative method

A ($1x10^8$ cfu/ml) suspension of Xcm isolate 2967 in SDW inoculated into susceptible cassava cultivar MCol 22 were used for this study. Two methods of inoculation were used.

1).to determine the spread in leaf- introducing 0.5ml of the bacterial suspension into the abaxial surface of the mid lobe of the third and fourth leaves of 2month old cassava plants by infiltration with a blunt sterile syringe. This resulted in watersoaked areas ca. 1.5x0.5cm per leaf.

2).to determine spread in stem-injection of 0.5ml of the bacterial suspension into two month old cassava stem at the base of the fourth leaf axil with the bacterial suspension using a sterile syringe with a needle.

The control plants were treated similarly with SDW. All plants were maintained in a Saxcil growth chamber. Four replicate stems or leaves were sampled every other day for each method of inoculation and the spread of the bacterium determined by plating leaf or stem discs on NYGA plates (Materials and Methods, 13a).

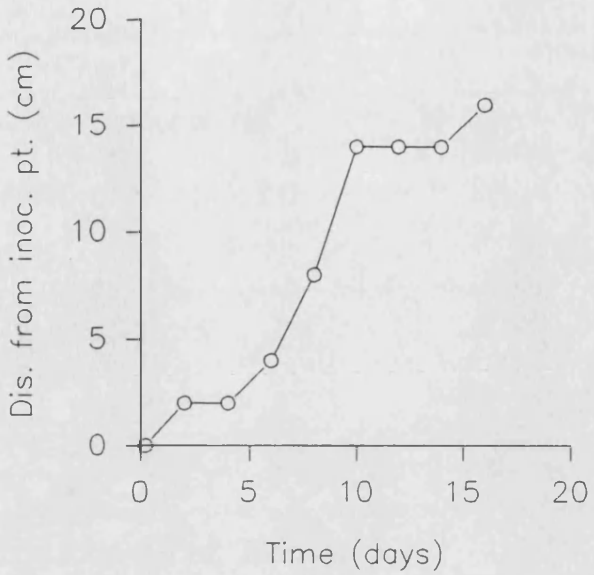
Two days after inoculation, bacteria could be reisolated from every lobe of the inoculated leaf although no symptoms were visible. By the time that the inoculated lobe of the leaf had become necrotic, bacteria had spread through the whole petiole (Fig.7). After about 18 days, the petiole withered completely and the leaf abscissed. No systemic spread of the bacterium or symptoms could be detected when the plants were inoculated in this method.

Plants inoculated by stem injection showed that the bacterium moved both upwards and downwards from the point of inoculation (Fig 8). Even after 5h bacteria could be detected about 3cm both up and down from point of inoculation (Fig.8) and after ca.6 days, the bacterial cells were present 9cm above and below the points of inoculation.

Some of the typical bacterial colonies that grew from the stem, petiole and leaf sections on NYGA plates (Plate 12) were reinoculated into healthy plants and they produced typical wilt and dieback symptoms further ensuring that the reisolated bacterium was Xcm.

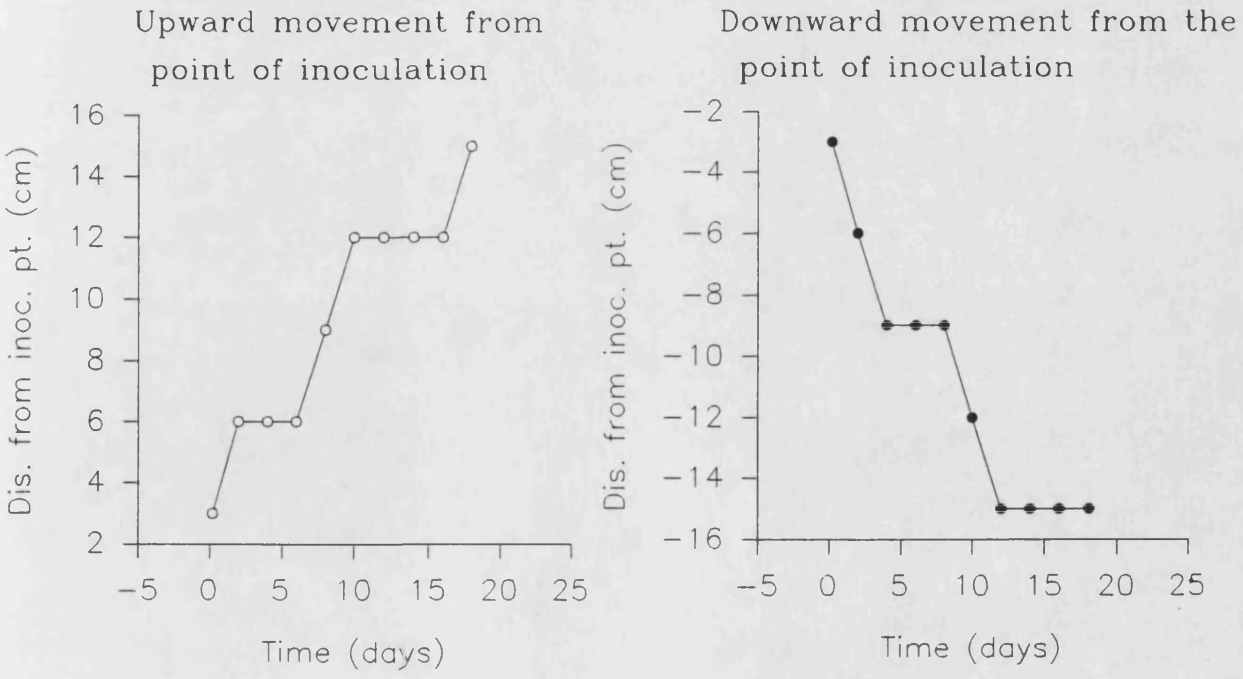
A common feature shown by plants infected by these methods was that the bacteria moved ahead before any symptoms were evident. This could indicate the necessity for the bacteria to multiply to a certain level to produce the required number of cells for symptom production or to produce a metabolite that is required to cause any visual damage to the host.

Fig. 7 Spread of Xcm isolate 2967 in petioles of susceptible cassava cultivar MCol 22 when infiltrated into leaves



Each point is an observation of four replicate plants.
All five lobes of the infiltrated leaf contained bacteria 2 days after inoculation.
6mm diameter leaf discs from each lobe and 2mm discs from every 3cm on the petiole was plated every other day.

Fig. 8 Movement of Xcm isolate 2967 in the stem of susceptible cassava cultivar MCol 22 inoculated by stem injection method



(ii).Quantitative method

In further studies, the multiplication of bacteria in the leaf was determined using quantitative reisolation techniques.

A suspension ($1x10^8$ cfu/ml)of Xcm isolate 2967 in SDW was inoculated into the third leaf of 2 month old cassava cultivars Mcol 22 and MNGA 1. Two methods of inoculation were used namely leaf infiltration and leaf clip method mentioned before (Section II,3i).

The control leaves were treated in a similar manner with SDW. Five replicate leaves were sampled every other day by grinding leaf discs and plating out dilutions on NYGA (Materials and Methods, 13b).

Bacterial numbers in the infiltrated lobe of both cultivars increased upto 10^{10} cfu/ml within 10 days (Fig. 9a) and remained high until the end of the experiment.

The bacteria also multiplied in the lobes on either side of the infiltrated lobe (Fig. 9b). In these lobes of both cultivars, bacteria increased upto ca.10^6 cfu/ml within 6 days and remained at this level until the end of the experiment.

Fig. 9a– Bacterial numbers in the infected lobe of two cassava cultivars inoculated with Xcm isolate 2967 using the leaf infiltration method

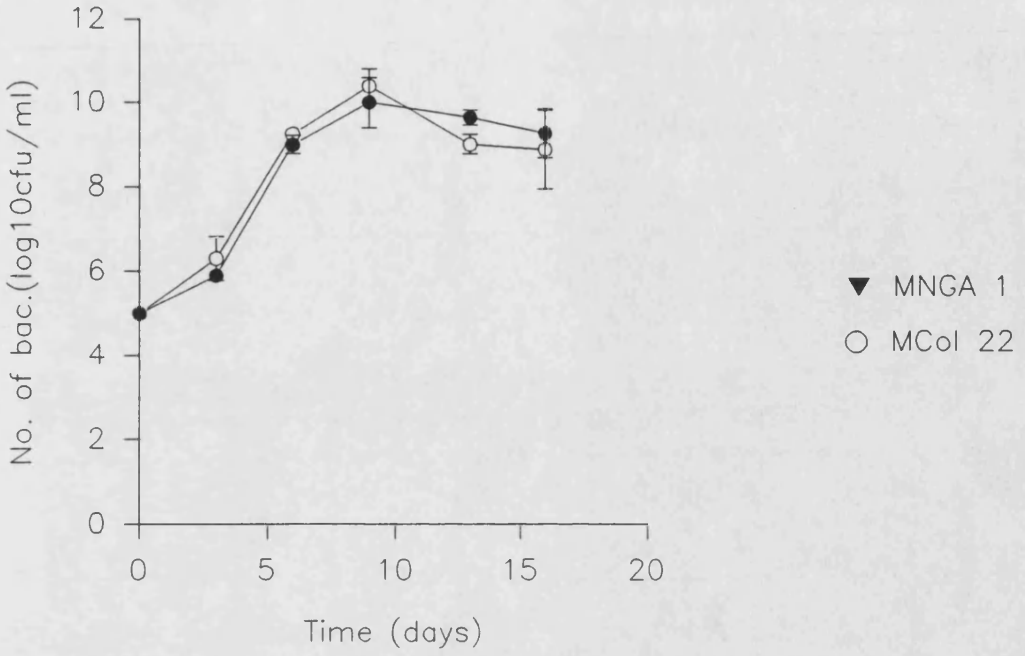
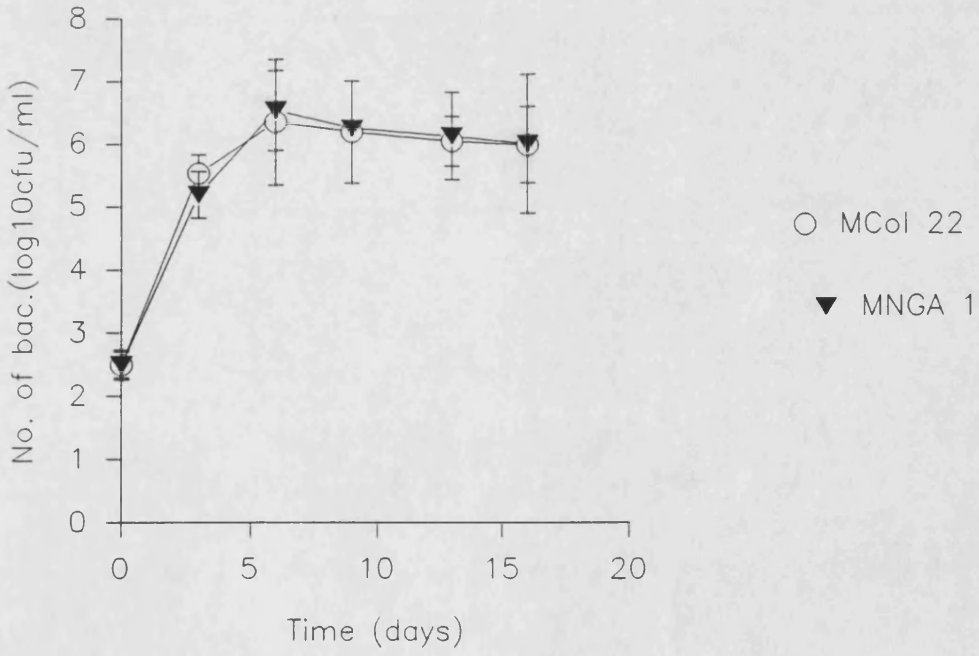


Fig. 9b– Bacterial no.s in the lobes adjacent to the infiltrated lobe of two cassava cultivars inoculated with Xcm isolate 2967



In both graphs vertical bars represent SD.

Each point in both graphs is a mean value of four replicate samples

Fig. 10a– Bacterial no.s 1cm from the infected surface of two cassava cultivars inoculated with Xcm isolate 2967 using leaf clip method

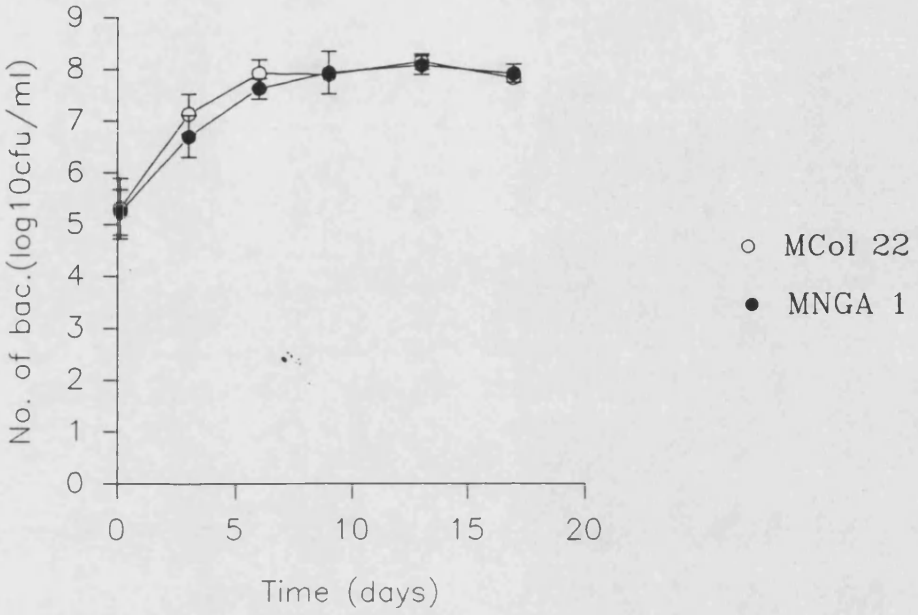
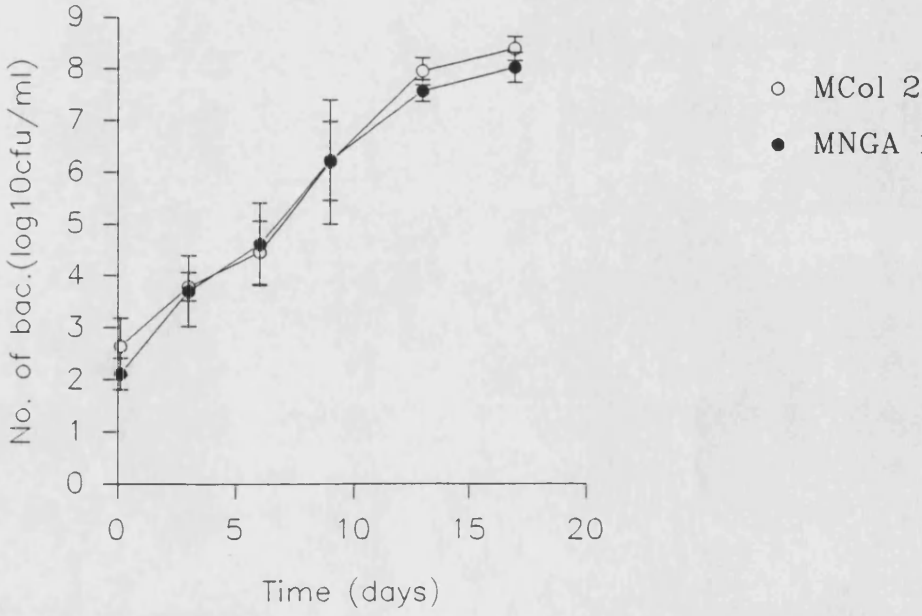


Fig. 10b– Bacterial no.s 6cm from the infected surface of two cassava cultivars inoculated with Xcm isolate 2967 using leaf clip method



Each point in both graphs is a mean value of fiive replicate samples.

In both graphs vertical bars represent SD.

Bacterial numbers were lower in the leaves inoculated by the leaf clip method as compared to leaves infiltrated with the pathogen. Hence, after 10 days ca 10^8 cfu/ml were detected using the leaf clip method as compared to ca 10^{10} cfu/ml in infiltrated leaves. There was no significant difference ($P \geq 0.05$, T test) in the bacterial multiplication in the two cultivars tested.

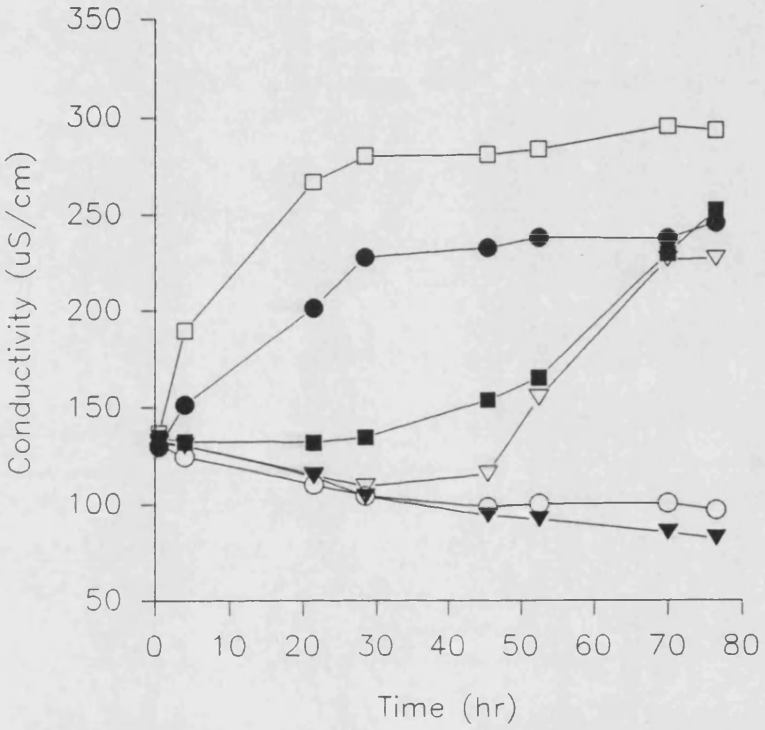
Spread of the bacteria from the cut edge showed that in both cultivars, ca 10^2 cfu/ml of bacteria cells were present 6cm from the clipped surface 4h after inoculation. Also bacterial numbers increased upto ca 10^8 cfu/ml in both cultivars within 16 days (Fig. 10a & 10b).

4. Effect of Xcm and Erwinia amylovora on electrolyte leakage from cassava leaves

The ability to induce host cell leakage by pathogens has been suggested as an indication of pathogenicity (Friedman & Jaffe, 1960; Wheeler & Hanchey, 1968; Cook & Stall, 1968; Brisset & Paulin, 1991). A cassava leaf disc assay was used in an attempt to perform a quantitative comparison of the responses in cassava cultivars rated as resistant or susceptible.

The effect of Xcm and Erwinia amylovora on the electrolyte leakage from leaf discs of cassava cultivars MCol 22, MCol 113, MNGA 1 and MVen 77 was tested (Materials and Methods, 11a).

Fig. 11–Effects of Xcm isolate 2967 and E. a. isolate 1430 on electrolyte leakage from leaf discs of susceptible (MCol 113) and resistant (MVen 77) cassava cultivars

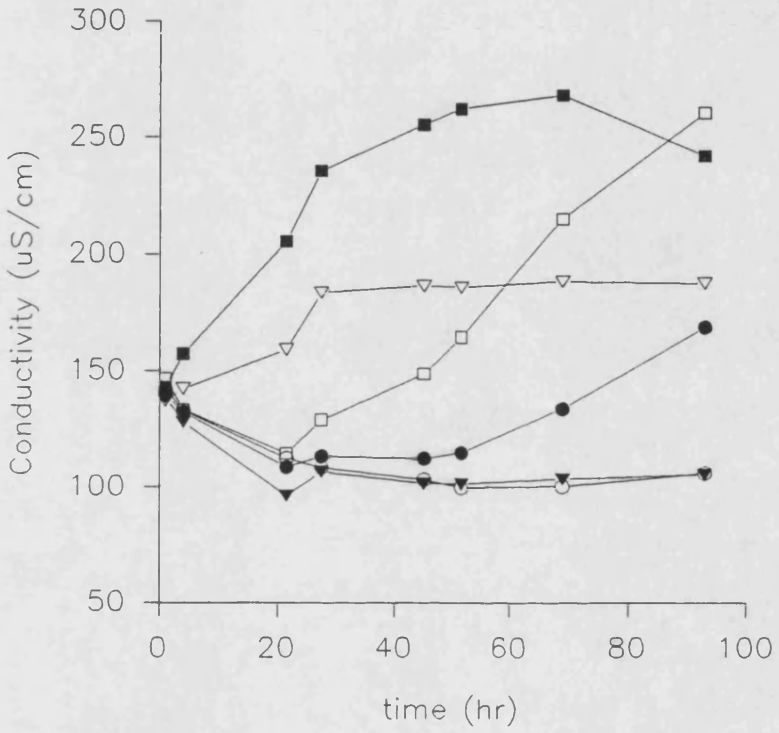


□ 113 infiltrated with E..a.
● 77 infiltrated with E. a.
■ 113 infiltrated with Xcm
▽ 77 infiltrated with Xcm
▼ 113 Control
○ 77 Control

SD between 1.0–6.11

Each point is a mean value of three replicate samples.

Fig. 12– EEffects of Xcm isolate 2967 and E.a. isolate 1430 on electrolyte leakage from leaf discs of susceptible (MCol 22) and resistant (MNGA 1) cultivars of cassava



- ■ 22 infiltrated with E.a.
- □ 22 infiltrated with Xcm
- ▽ MNGA 1 infiltrated with E.a.
- ● MNGA 1 infiltrated with Xcm
- ▼ 22 Control
- ○ MNGA 1 Cont.

SD values between 1.0–6.14

Each point on the graph is a mean value of three replicate samples.

Table 20- Effect of Xcm isolate 2967 and E.a. isolate 1430 on electrolyte leakage from susceptible and resistant cassava leaf discs

		Conductivity(uS/cm) after incubation		
Cultivar	Treatment	20h	45h	70h
MNGA 1	Xcm	108.3±3.06(a)	112.3±6.11(a)	133.67±1.2(c)
	E.a.	159.0±3.61(b)	186.3±4.04(b)	188.3±5.1(b)
	Control	112.0±2.0(a)	103.0±2.65(a)	100.0±1.0(a)
MCol 22	Xcm	114.3±3.06(a)	148.7±4.62(c)	215.0±5.0(d)
	E.a.	205.3±6.14(b)	255.3±3.43(b)	268.0±3.0(b)
	Control	96.0±1.0(a)	101.3±5.13(a)	103.3±2.5(a)

Leaf discs of cassava cutivars MNGA 1 and MCol 22 were infiltrated with Xcm, E. amylovora or MES buffer (as a control). Values are a mean of three replicates±SD. For each cultivar, values within a column followed by the same letter are not significantly different at P=0.05 (ANOVA followed by Bonferroni's test).

After 20h leaf discs of all four cassava cultivars infiltrated with Erwinia amylovora showed a significantly higher electrolyte leakage ($P<0.05$) as compared to the controls and discs infiltrated with Xcm (Figs.11 and 12). The electrolyte leakage from these discs continued until complete electrolyte loss.

The level of electrolyte leakage in the control tissues remained almost constant throughout the

experiments although decrease in leakage was observed with MCol 113 and MVen 77 with extended incubation, it was not significant.

The electrolyte loss from discs of susceptible cassava cultivars i.e. MCol 113 and MCol 22 infiltrated with Xcm isolate 2967 was significantly higher ($P<0.05$) after 45h whereas the electrolyte loss from discs of resistant cultivars i.e. MNGA 1 and MVen 77 did not start increasing upto ca. 55-70h (Table 20, Figs. 11 & 12).

DISCUSSION II

When the pathogenicity of Xcm isolate 3194 was tested on the susceptible cultivar MCol 22, it was shown that stabbing or injecting the stem with the bacterial suspension resulted in typical symptom production (leaf wilt, leaf abscission, stem dieback and gum exudation). However, spraying the plants with a bacterial suspension or injecting the bacterial suspension into the petiole induced only a limited response. Symptoms were restricted to the points and areas close to inoculation and following necrosis and wilting the leaves dropped off without the bacteria moving into the stem. A response of this nature to restrict the movement of the bacterium would be associated with resistance of the host plant to the pathogen. Lozano (1975) has made similar observations when susceptible cassava plants were inoculated by spraying the Xcm supension or by rubbing the leaves with a cloth dipped in the Xcm suspension. Symptoms did not spread from the areas inoculated indicating that bacterial movement from leaves to the stem was restricted. Also, Maraite & Meyer (1975), in an investigation of cassava bacterial blight in Zaire, reported that when cassava leaves were infected they generally dropped off before the stem is invaded. This was contrary to the report of CIAT (1975) where leaf inoculation resulted in the systemic spread of the

bacteria and stem dieback occurred.

All the Xcm isolates tested here with the exception of isolate 2444 were highly virulent and caused severe disease symptoms on cassava. The apparent lower virulence of isolate 2444 could be due to its slower growth rate as observed on NYGA plates rather than to it being less virulent.

The syndrome induced by Xcm on cassava is complex and appears to consist of two phases; the leaf phase where leaf spotting, necrosis and wilt occurs and the systemic phase where shoot wilting, stem dieback, gum exudation and vascular discoloration has been observed. Similar symptoms of leaf spotting, shoot wilting, stem dieback, gum exudation and and vascular discoloration has been observed with pseudomonad pathogens of deciduous fruit trees (Crosse, 1966). Cassava bacterial blight disease involves two phases of natural infection. In the first method natural infection is facilitated through rain splash which results in bacterial penetration through stomates (Lozano & Sequeira, 1974a; Lozano, 1972) and epidermal wounds (Pereira & Zagatto, 1967). In this study, this invasion route was simulated by spraying or infiltration of the leaves with the bacterial suspension or clipping the host leaves with scissors dipped in the bacterial suspension. Alternatively, natural infection may involve planting infected cuttings

whereby the bacteria would be already present in the vascular system of the host plant (Lozano & Sequeira, 1974b). Artificial inoculation by injecting the bacterial suspension into the host stem with a syringe fitted with a needle could be assumed to simulate this natural infection.

These symptom patterns could be the result of numerous factors including the production of macromolecules such as bacterial EPS. EPS is considered to contribute to wilting of plants by vascular occlusion (Ayers et al., 1979; Bradshaw-Rouse, et al., 1981). Duvick & Sequiera (1984) have claimed that virulence of a pathogen is a function of EPS production since rough mutants lacking EPS were avirulent. However, Xu et al. (1990) using TN5 mutagenesis of Pseudomonas solanacearum, obtained a class of mutants which had lost the ability to produce EPS but retained wild type virulence to tobacco and eggplant. EPS was observed to occlude xylem vessels of cassava in the present study (Section IV) and thus could be one of the factors causing the wilt symptoms.

Pathogen produced toxic metabolites also have been reported to cause water loss and thus induce plant wilting by affecting the host in various ways such as opening of the stomates irreversibly (Marrë, 1979) or affecting membrane permeability (Pegg, 1989). However, damage to membranes was not seen in TEM investigations in

the present study.

The xylem fluid as a substrate is relatively low in nutrients (Pegg, 1981a) and the production of extracellular enzymes may represent a means of providing the pathogen with essential or supplementary nutrients by degrading and removing carbon fragments from the pit membrane (VanderMolen et al., 1987). The degradation of pit membranes would also facilitate the movement of bacteria between vessels. Although Xcm was shown to produce pectate lyase in vitro, it was not detected in infected plants (Section III). However, very low levels which could be sufficient for dissolving pit membranes might be produced thus facilitating the movement of Xcm between vessels.

In this study, the speed of spread and multiplication of Xcm in the host plant was investigated both qualitatively and quantitatively and this is the first study in quantifing multiplication of Xcm in host tissue. It was found that the bacteria were present in host tissues well ahead of symptom production. It is possible that a critical minimum of bacterial cells have to be present in host tissues before disease symptoms appear. Similarly, Robinson & Callow (1986) isolated Xanthomonas sp. from well ahead of the leaf tips of infected Brassica, Oryza and Phleum leaves where the HR response was visible.

Xcm moved approximately 3cm from the inoculation point both upwards and downwards when the inoculated plants were sampled after only 5h. This speed of bacterial spread might indicate that movement in the vascular system occurs; this trend continued with Xcm detected at 9cm from point of inoculation within 8 days and 12cm within 10 days. Lozano (1972), Lozano & Sequiera (1974), Amaral (1942) and Drummond & Hipolito (1941) have suggested systemic movement of Xcm in cassava plants and the discoloration of vascular tissues could also be an indication of this. It can be assumed that the movement takes place through the xylem and this was confirmed by TEM studies (Section IV). Zimmermann (1983) has suggested that a bacterium penetrating a vessel would produce a hole in the vessel wall which would be large enough to cause cavitation of the xylem transpiration stream. Thus the entering bacterium could then be swept either up or down to the end of the vessel where it would lodge against the next vessel-to-vessel pit membranes, multiply in the humid space of the vapour filled vessel and eventually enter the next vessel destructively. This may explain why colonization was observed down from the inoculation point on stem as well as above.

The quantitative study of the 'leaf phase' of the disease showed that the bacteria were present in the

lobes on either side of the inoculated lobe after 4h. This speed of movement would again might indicate that they might be moving in the vascular system and bacteria were observed exclusively in xylem tissues leaves by TEM (Section IV).

Inoculation of bacteria at densities of 10^8 and 10^9 cfu/ml is common practice in bacterial plant pathology but is an artificial process. However, although such inocula may swamp otherwise effective constitutive resistance mechanisms, induction of an active plant response is often more obvious when high density inocula are used (Klement & Lovrekovich, 1961; Ercolani & Crosse, 1966; Young, 1973; Lyon & Wood, 1976). In this study, leaf infiltration and inoculation by leaf clip were carried-out with bacterial suspensions with 10^8 and 10^9 cfu/ml. Inocula of bacteria at this density were well above the threshold necessary to cause disease in every inoculated plant.

An experiment carried out to test the effect of the bacterial inoculum concentration on the expression of resistance indicated that inoculum levels as low as 10^2 cfu/ml produced symptoms in susceptible cultivars after 12 days but failed to do so in the resistant cultivars. It was also shown that the larger the initial number of bacteria introduced, the shorter was the period necessary for incubation. Weindling (1948), from work with

artificial inoculation of X. malvacearum in cotton, reported a similar phenomenon.

In leaves inoculated by both methods, the bacterial numbers were very slightly less but not significantly different in the cultivar rated as resistant as compared to the susceptible cultivar upto 3 days. After 3 days the bacterial numbers remained at a similar high level in both cultivars tested.

The only apparent difference in symptom development between resistant and susceptible cultivars was a slight delay, 1-2 days, in the resistant cultivars. According to CIAT (1975), the leaf clip method resulted in stem die-back in susceptible cultivars, but this was not seen in this study. Therefore, the similarity in symptom development in resistant and susceptible cultivars could be due to the fact that the susceptible cultivars did not allow complete disease development when inoculated in this manner but this seems unlikely. Alternatively, the similarity could be due to the fact that the resistant cultivars show a tolerance rather than a true resistance towards the pathogen so that the symptom development is delayed for a short period until the bacterium overcomes the effects of this reaction. The conditions under which resistance to Xcm was tested was highly favourable to the pathogen. For this reason, the resistant cultivars could be expected to exhibit a greater degree of tolerance in

the field. However, Lozano & Sequeira (1974b) have reported of varietal resistance to Xcm under glass-house conditions which confirmed field observations (Carneiro, 1940; Gonclaves, 1939,1948; Periera & Zagatto, 1967). These workers showed that most resistant cultivars had a significantly lower number of leaf spots per leaf than the susceptible cultivars. Studies by Lozano & Sequiera (1974b) have also hypothesised that three possible types of resistance exist in cassava cultivars; a first type limits penetration, another limits systemic invasion and the third based on the hypersensitive reaction of the host. However, in contrast, in a study of cassava bacterial blight in Zaire, Maraite & Meyer (1975) failed to detect any varietal resistance in cassava.

According to IITA in Nigeria and CIAT in Colombia resistance of cassava to Xcm is multigenic (Lozano, 1986) and therefore it could be argued that under the conditions used in the present study, the genes required for complete resistance were not expressed and could be influenced by a number of environmental factors such as temperature. Webster et al. (1983) reported a similar effect on bean infected with X.campestris pv phaseoli. They showed that bean cultivars Jules and PI 207262 were moderately resistant to X.c. phaseoli in the temperate zone but susceptible in the tropics. Arnold & Brown (1968) also demonstrated the importance of environment in the expression and selection of polygenic resistance to

bacterial blight of cotton caused by X. malvacearum. In Uganda they were able to recover levels of resistance following several generations of inbreeding in material that had formerly been found susceptible in Sudan. Also the SR6 gene for resistance to stem rust from the wheat variety Red Egyptian is temperature sensitive. At 20°C it confers resistance to an avirulent race of Puccinia graminis but at 25°C it gives a susceptible reaction (Loegering & Geiss, 1957).

In this study, the disease ratings given by Lozano & Laberry (1982) were used and cultivars MCol 22 and MCol 113 proved to be as susceptible to Xcm as claimed by their studies. However, Joseph & Elango (1991) in work carried out in Trinidad report that MCol 22 was moderately resistant in the field although susceptible in the glass-house. This may also indicate that environmental conditions may be involved in the variability in disease responses in cassava towards Xcm.

The effect of Xcm isolate 2967 and Erwinia amylovora on resistant and susceptible cassava cultivars was compared by measuring the loss of electrolytes from inoculated leaf discs. E. amylovora caused increased electrolyte loss from both resistant and susceptible cultivars within a short period of time which could be the result of a HR reaction as this bacterium is nonpathogenic to cassava. Xcm was slower to cause

electrolyte loss from susceptible leaf discs and even slower in the resistant. This was in agreement with the symptom production by Xcm in whole cassava plants. The longer time required by the bacterium to react with the resistant cassava cultivars was reflected in the slight delay (55-70h) in causing increased electrolyte loss from those discs. If a hypersensitive-based resistance had been shown, a rapid loss of electrolytes similar to that induced by E. amylovora should have been evident.

The increase in conductivity of susceptible plant tissue following death or injury is attributed to increased permeability of the semipermeable membranes of the tissue cells permitting leakage of ions to the intercellular spaces. Such damage could be caused by toxins or enzymes produced by the pathogen. Friedman & Jaffe (1960) showed that inoculation of chicory tissue with living cells or culture fluids of Erwinia and Pseudomonas spp. resulted in increased tissue conductance. Similarly, Cook & Stall (1968) showed a difference in the increase of conductance between resistant and susceptible pepper varieties inoculated with X. vesicatoria. Brisset & Paulin (1991) showed that one of the first events associated with interaction between a necrogenic bacterial pathogen E. amylovora and cells of a susceptible (pear) plant is the leakage of electrolytes and nutrients through plant cell membranes.

The method followed to introduce bacteria into leaf tissues in this method is perhaps artificial and may result in higher or faster membrane damage indicated by electrolyte loss could be higher than in a diseased plant. Indeed, ultrastrucural study of infected leaves did not show clear membrane damage in diseased host cells (Section IV) but perhaps in the early stages biotrophic pathogens can cause increased membrane permeability without causing membrane disruption.

In summary, Xcm isolates caused typical disease symptoms of cassava bacterial blight under the conditions tested. However, the 'leaf phase' did not develop into a stem phase as indicated by some workers. Although the resistant cultivars supplied by CIAT showed a slight delay in symptom production and reduced electrolyte leakage, bacterial multiplication was not affected. Therefore, in the absence of clearcut resistance a study of the mechanisms of resistance was not continued further.

SECTION III- PATHOGENICITY DETERMINANATS OF Xcm

Xcm must enter, obtain nutrients from host cells presumably by damaging them, multiply, and spread in the host tissues. In view of these concepts, and the symptomatology of the disease, its systemic nature and claims of other workers on Xcm and other xanthomonads (Perreaux et al., 1982; 1986; Ewbank & Maraite, 1990; Lakshmanan & Pandian, 1988; Starr & Nasuno, 1967; Dye, 1960; Abo-El-Dahab, 1964; Dow et al., 1989; Beaulieu et al., 1991; Ikotun, 1984a), possible involvement of a toxin and a pectolytic cell wall degrading enzyme was investigated. A study of the nature of pathogenicity factors of Xcm was conducted following growth on a range of media in vitro and in vivo.

A). Investigation of the possible involvement of a toxin in pathogenicity

Certain spp. in most genera of phytopathogenic bacteria have been reported to produce toxic metabolites (Mitchell, 1981) and some (eg. Pseudomonas syringae) have been studied extensively. Members of the genera Xanthomonas, Erwinia and Corynebacterium have all been reported to produce toxic metabolites, but the role of these compounds in disease development has not yet been clearly demonstrated. The possibility of a toxin being involved in the pathogenesis of cassava by Xcm was

investigated because the chlorotic lesions that spread from necrotic centres could be similar to the symptoms of halo blight on Phaseolus vulgaris for which a toxin from P. syringae pv phaseolicola is clearly implicated and also because previous workers have claimed the production of a toxin by Xcm.

One possible benefit of proving the involvement of a toxin would be that it could be used instead of the bacterium as a selection pressure for identifying resistant plants from in vitro cultures, if regeneration could be achieved. Such systems have been used successfully by other workers to regenerate disease resistant plants (eg. Brettel et al., 1979; Behnke, 1979; Sacristan, 1982; Hammerschlarg, 1988; Nachmias, 1990). The study of the possible involvement of a toxin would also give an insight into the mode of pathogenesis of Xcm.

Initially, the production of the toxin under various culture conditions was monitored using different bioassays.

1. Bacterial growth in different concentrations of Minimal medium B (Mm B)

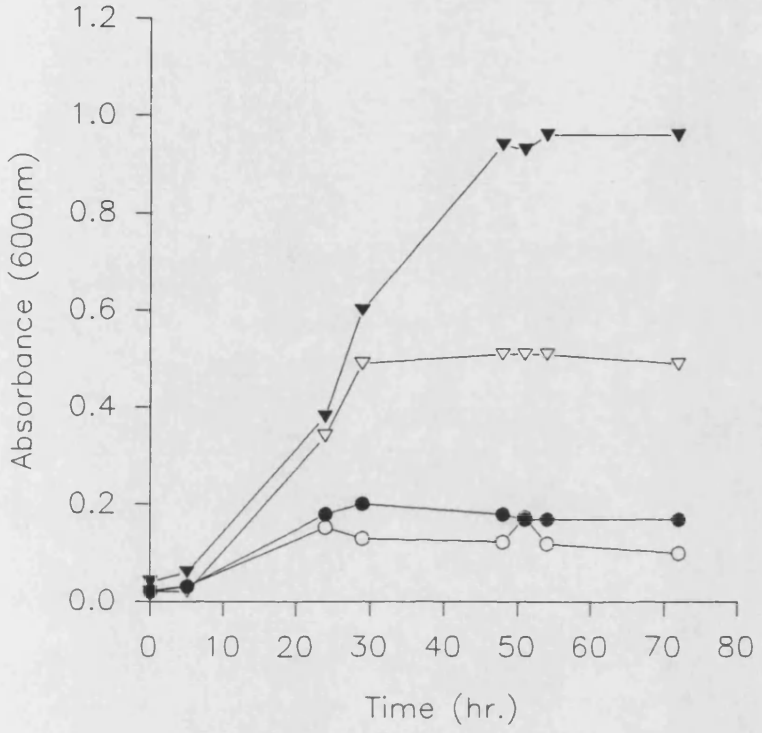
In order to obtain cell free culture fluids for assays for toxicity it is preferable to grow bacteria in a defined basal medium than in a complete medium. Growth

in minimal media should lessen the presence of extraneous medium components which may reach phytotoxic levels on concentration of fluids prior to bioassay. An inherent problem with the use of concentrated culture fluid is the possible toxicity resulting from concentration of spurious metabolites or of culture salts (eg. Buchanan & Starr, 1980). In an attempt to test whether the salts in the minimal medium used here could be further reduced without hindering mutiplication of the bacterium, growth of Xcm isolate 3194 was determined in 1/10, 1/5, 1/2 concentrations and full strength minimal medium B (see Appendix I for details).

5 ml of the bacterial suspension($1x10^8$ cfu/ml) was added to 45 ml of the above mentioned concentrations of minimal medium B to give a final concentration of $1x10^7$ cfu/ml. Methionine was added at 10ug/ml to all concentrations of minimal medium.

The growth on 1/2 and full strength Mm B was almost the same upto about 30 hrs, after which the growth on the full strength medium was considerably higher (Fig.13). Growth on 1/10 and 1/5 concentration of the minimal medium was markedly less throughout, therefore, the full strength Mm supplemented with 10µg/l methionine was used for further experiments on toxin production.

Fig. 13-- Growth of Xcm isolate 3194 in different concentrations of Minimal medium B



○ 1/10 concentration of Mm B

● 1/5 concentration of Mm B

▽ 1/2 concentration of Mm B

▼ full strength Mm B

Methionine was added at 10ug/ml

Each point in the graph represents the mean value of four replicates.

SD 0.01-0.025.

Cell free culture extracts of isolates 3194 and 2967 were obtained after 36 and 48h and assessed for toxicity.

2.Use of diffusion capsules to grow Xcm under restricted culture conditions

Diffusion capsules were used in a further attempt to control the supply of nutrients to bacterial liquid cultures (i)in an attempt to overcome the toxicity of salts and sugars in culture fluids if the fluids need to be concentrated and (ii)to prevent catabolite repression which may regulate production of pathogenicity factors (Cooper & Wood, 1975). After sterilisation by autoclaving at 121°C and 1.4Bars for 15 mins, the capsules containing the appropriately concentrated nutrients were placed in shake cultures. Rates of release of nutrients from capsules were altered by changing the nutrient concentration, altering the number of capsules per culture and by changing the number of membranes in capsules.

2(a).Determination of ion diffusion rate from capsules

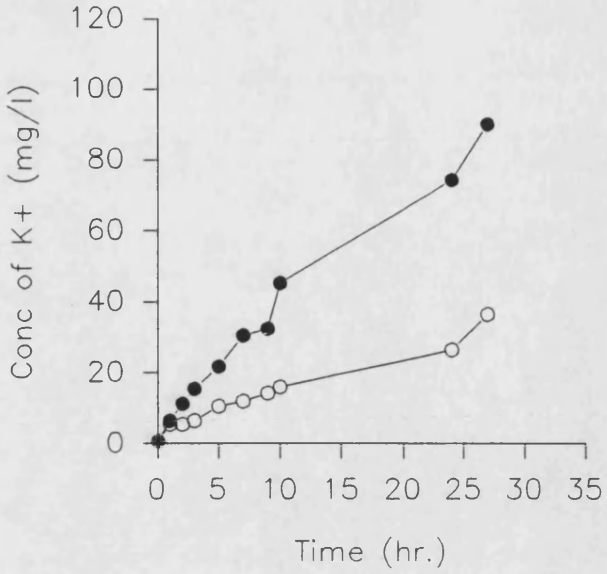
The salts of minimal medium B (except for $CaCl_2$ $2H_2O$ and $MgSO_4$) were added at 10 and 20 times concentration of that provided in Mm B and placed in separate diffusion capsules. Glucose was added at x30 concentration of that provided in Mm B and added to separate capsules. Two

membranes were used in each capsule. $CaCl_2 2H_2O$ and $MgSO_4$ could not be added together into diffusion capsules as they would not dissolve when concentrated, therefore they were added at the normal concentrations as in minimal medium B to 100ml of sterile deionised water in 250ml flasks. A capsule with 10 or 20 times concentrated salts and another containing the concentrated glucose solution were added to each flask which was rotated at 150rpm at 30°C.

One ml aliquots were removed at various time intervals and tested for K^+ and glucose concentration with a flame photometer (Corning 400) and a glucose oxidase kit (Sigma) respectively to determine rates of diffusion.

Fig 14 shows the rates of diffusion over 27h of K^+ as an indicator of other medium salts and Fig. 15 shows the rate of diffusion of glucose over the same time. These conditions allowed relatively linear rates of release of salts and sugars over 24h so that the capsules required changing only daily to support bacterial growth. The levels after 27h of K^+ (40-90mg/l) and glucose (175mg/l) are markedly lower than in the original medium (625mg/l and 5000mg/ml respectively).

Fig.14– Rate of release of K +from diffusion capsules



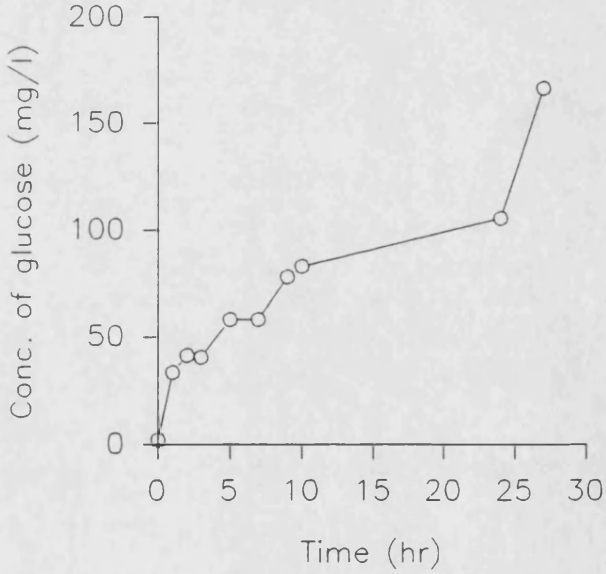
o diffusion of K+ into 100ml water from capsules containing x10 concentrated Mm B

• diffusion of K+ into 100ml water from capsules containing x20 concentrated Mm B

Level of K + in minimal medium B= 625mg/l

K+ levels were measured using a Corning 400 flame photometer.

Fig. 15– Rate of release of glucose from diffusion capsules



o diffusion of glucose from capsules (glucose concentratedx30 cf.. Mm B)

Level of glucose in Mm B= 5000mg/l

Glucose levels were measured using a glucose oxidase kit. (Sigms 510–DA).

Each point in both graphs represents a mean value of four replicates.

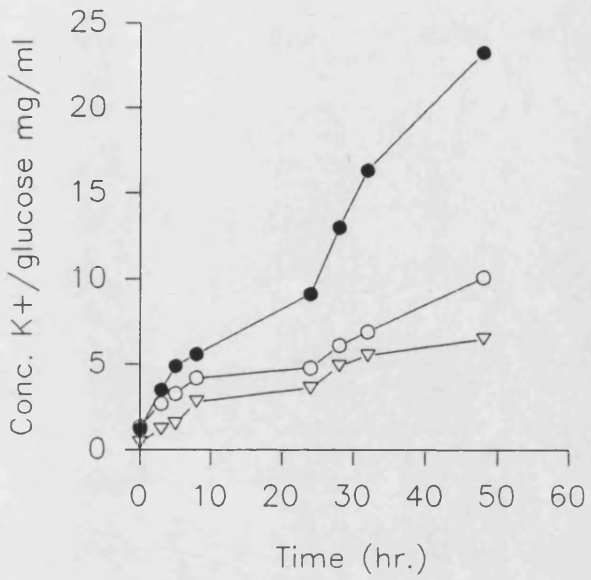
2(b).Restricted supply of nutrients

As the conditions used in the previous experiment gave satisfactory diffusion, allowing linear rates of release of glucose and salts over a period of 27h, these were used for the growth of bacteria to obtain culture filtrates.

A standardised inoculum was prepared by centrifuging (13000g, 15 min.) overnight cultures of Xcm isolates 3194 and 2967 in 100ml of NYGB and resuspending in 100ml of $CaCl_2 2H_2O$ + $MgSO_4$ solution.Two diffusion capsules were added to each flask so that each flask contained either a capsule with the salts concentrated 10 times + a capsule with glucose concentrated 30 times or a capsule with the salts concentrated 20 times + a capsule with glucose concentrated 30 times. The flasks were incubated in a rotary shaker (150 rpm) at 30°C. Fresh capsules were added in the same manner after 24h. Samples were obtained from these cultures at time intervals and K^+ and glucose concentrations were measured as described earlier.

Cell free culture fluids were obtained after 36 and 48h of growth as described in Materials and Methods,8. Fig. 16 shows the levels of K^+ and glucose present in the cultures. After 30h K^+ levels were 5-7mg/l and glucose about 17mg/l. Growth of Xcm reached late log phase after about 10h and then remained apparently stationary in restricted culture (Fig. 17).

Fig. 16– Concentration of K+ and glucose in cultures of Xcm isolate 3194 growing under restricted culture conditions



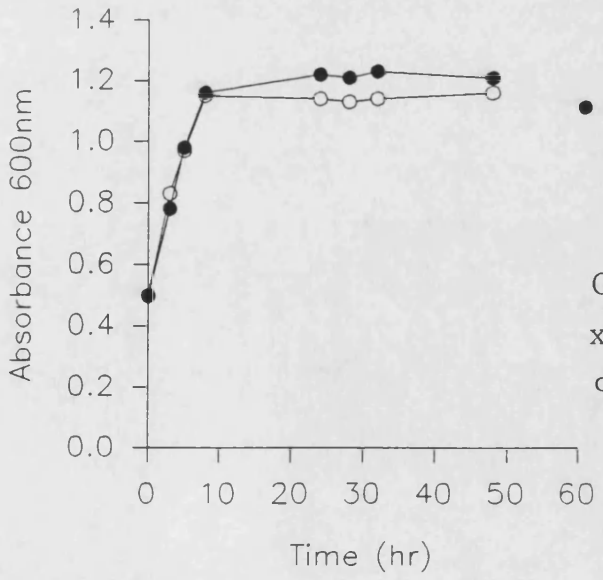
● glucose concentration in cultures with capsules containing glucosex30

○ K+ conc. in cultures with capsules containing salts x20

▽ K+ conc. in cultures with capsules containing salts x10

K+ levels were measured with a Corning 400 flame photometer and glucose levels with a glucose oxidase kit (Sigma 510–DA) Fresh capsules were added every 24h.

Fig. 17– Growth of Xcm isolate 3194 under restricted culture conditions



○ growth of the bacterium with diffusion capsules containig x10 concentrated nutrients

● growth of the bacterium with diffusion capsules containing x20 concentrated nutrients

Glucose was concentrated x30 and added in a separate capsule

Each point in both graphs represents the mean value of four replicates

3.Assay of culture fluids for toxicity

3(a).Assay of culture fluids obtained by growing Xcm isolate 3194 for 36 and 48h in Mm B

Three bioassays were performed with the cell free culture fluids of Xcm isolate 3194. ie (i)infiltration into the abaxial surface of healthy leaves of susceptible cultivar M Col 113 to induce macroscopic symptoms, (ii)effect on viability of 20d suspension cultured cells of susceptible cultivar M Col 113 and (iii)estimating the ion leakage from leaf and stem discs of susceptible cassava cultivar M Col 113 (details in Materials and Methods,13).

Leaf bioassay Leaves infiltrated with either 36hr or 48hr culture fluids showed no effect other than faint brown discoloration at the point where the syringe was adpressed during infiltration of the culture fluid; the controls that consisted of leaves infiltrated with the uninoculated medium showed a similar effect.

Suspension cultured cell bioassay No significant change in viability of 20d old suspension cultured cells of cultivar M Col 113 was caused by culture fluids. Thus when the 36 h culture fluid was added to the cells, they maintained a viability of 64.60±2.5% after 5 days compared to the 63.78±1.2% viability of the cells of the controls (Table 21). Similarly, the cells showed no

significant change in viability with the 48 hr culture fluid.

Ion leakage bioassay Stem and leaf discs of susceptible cultivar MCol 113 were added separately to 36 and 48 h culture fluids of Xcm isolate 3194 grown in Mm B, and tested for loss of ions. Table 22 shows that there was no significant change in the release of K^+ from treated leaf discs compared to the controls indicating that there is no apparent damage to cell membranes.

Similarly, the 36 and 48hr culture fluids of Xcm isolate 2967 grown in Mm B showed no toxic effect when tested using the leaf infiltration assay.

3(b).Assay of culture fluids obtained by growth of Xcm isolates 3194 and 2967 under restricted culture conditions

The effect of culture fluids obtained after 36 and 48h of growth of Xcm isolate 3194 under restricted culture conditions was tested using the three bioassays and toxicity of fluids from isolate 2967 were examined by leaf infiltration bioassay only.

No toxicity was observed by any of the bioassays (Tables 23 and 24).

Table 21- The effect of cell free culture fluids Xcm isolate 3194 grown on Mm B for 36 and 48hr of on the viability of 20d old suspension cultured cells of cassava cultivar M Col 113

time (days)	% viability of cells ± SE		
	control	treated (36h)	(48h)
0	78.00±1.2	78.00±1.8	75.00±1.5
1	76.80±0.8	72.50±1.2	74.87±0.9
2	72.30±2.4	73.40±0.7	72.60±0.6
3	70.60±1.7	68.50±1.3	70.00±1.3
4	66.00±0.9	65.89±0.8	67.00±1.3
5	63.78±1.2	64.60±2.5	66.8±0.8

Every reading is a mean of four replicate samples.

Cell viability was estimated by using the vital dye fluorescein diacetate.

Table 22 -The effect of cell free culture fluids of Xcm isolate 3194 (grown for 36 h in Mm B) on K^+ leakage from leaf and stem discs of cultivar M Col 113

time(h)	K^+ level (mg/l) stem discs control	stem discs treated	leaf discs control	leaf discs treated
0	43.00±1.5	41.60±1.7	43.00±1.4	43.30±1.0
3	46.00±2.1	40.66±1.4	40.00±1.6	40.33±1.8
5	39.00±1.9	37.30±1.2	42.00±1.6	37.30±1.4
15	45.00±1.3	37.30±1.4	40.00±1.6	41.30±1.5
18	41.00±1.0	40.00±1.5	43.00±1.5	37.00±1.2
24	45.00±2.3	41.66±1.6	42.00±1.4	40.30±0.9
26	44.00±1.8	37.60±1.2	38.00±1.3	35.30±1.6
28	40.00±1.4	39.00±0.8	46.00±2.1	35.30±1.3
45	40.00±1.6	38.60±1.5	43.00±1.3	37.00±1.3
47	42.00±1.5	37.30±1.4	36.00±1.4	34.00±1.6
49	45.00±1.3	40.66±1.5	36.00±1.2	31.66±1.4

All readings are mean values of four replicate samples.

Culture fluids obtained after 48h gave essentially the same results.

Table 23- The effect of cell free culture fluids of Xcm isolate 3194 (grown for 36h under restricted culture conditions) on K^+ leakage from stem and leaf discs of cassava cultivar M Col 113

time(h)	concentration of K^+ (mg/l) A				B			
	stem discs con.	treat.	leaf discs con.	treat.	stem discs con.	treat.	leaf discs con.	treat.
0	8.0	10.0	11.5	10.5	19.0	20.0	17.0	16.0
4	9.5	8.6	9.6	10.7	17.8	17.5	18.6	19.0
8	8.7	7.9	8.5	7.9	18.8	18.5	20.6	19.8
15	7.9	10.0	7.3	8.5	20.6	19.8	18.7	20.2
18	9.6	8.7	8.9	9.1	19.0	18.2	20.1	17.9
24	8.5	9.5	7.8	8.1	18.4	21.6	20.5	19.0
28	7.8	8.1	9.0	7.9	17.9	18.3	18.5	19.0
36	7.5	7.6	8.7	7.5	18.9	18.4	19.3	18.2

A- cell free fluid from the cultures grown with diffusion capsules with 10 times concentrated salts

B- cell free fluid from the cultures grown with diffusion capsules with 20 times concentrated salts

con.- control treat.- treated

Table 24- The effect of cell free culture fluids of Xcm isolate 3194 (grown for 48h under restricted culture conditions) on K^+ leakage from stem and leaf discs of cassava cultivar M Col 113

	concentration of K^+ (mg/l)							
time(h)	A				B			
	stem discs		leaf discs		stem discs		leaf discs	
	con.	treat.	con.	treat.	con.	treat.	con.	treat
0	8.8	9.0	8.5	7.5	18.0	21.0	17.0	18.2
4	7.5	8.8	7.6	8.7	19.8	17.5	20.6	24.0
8	10.7	9.5	8.5	8.9	22.8	24.5	20.6	19.8
15	9.9	10.0	10.3	9.5	20.6	22.8	24.7	20.2
18	10.6	9.8	11.5	8.9	19.0	18.2	20.1	23.9
24	9.5	9.5	12.8	9.1	23.4	21.6	20.5	24.0
28	8.8	10.1	9.0	10.9	22.9	21.3	23.5	19.0
36	10.5	9.8	10.7	11.5	24.9	25.4	19.3	21.2

A- cell free fluid from the cultures grown with diffusion capsules with 10 times concentrated salts

B- cell free fluid from the cultures grown with diffusion capsules with 20 times concentrated salts

In summary, cell free culture fluids were obtained from two different stages of the growth cycle of the bacterium, i.e. early log and late log, by growing two Xcm isolates (3194 and 2967) under two culture conditions. Each of these experiments were repeated twice. All these criteria failed to reveal any toxicity

to cassava cells of culture fluids by the methods tested. Therefore, it can be concluded that Xcm does not produce any toxic metabolites under this range of conditions or, if any were produced, they were unable to show any effect under these conditions. The use of three very different bioassays should have precluded the last possibility.

4.Effect of growth of Xcm in Watanabe medium

As the culture fluids obtained from Xcm liquid cultures grown under the previous culture conditions failed to show any toxicity, the method used by Perreaux et al. (1982) was followed because they claimed to have demonstrated toxin production by Xcm. Bacterial isolates 3194 and 2967 were grown in 100ml of modified Watanabe broth (Watanabe (1963) see Appendix I for details) in 250ml flasks in a rotary shaker (150 rpm) at 28°C for 6 days. Culture fluids were either used directly or following the extraction protocol of Perreaux et al. (1982). For the extraction culture fluid was centrifuged for 15 min. at 13000g, concentrated to 1/3 of its original volume by evaporation at 40°C under vacuum and adjusted to pH 4 with 1N HCl. Ethyl acetate was added (1:1, v/v) and the mixture shaken for 5 min. This was repeated twice, then the combined ethyl acetate fractions were completely evaporated and the residue taken up in sterile water to give a 40 times concentration and filtered through 0.2µm millipore filter.

The extract was diluted x10, $x10^{-2}$ and $x10^{-3}$ and tested for toxicity using assay 1. Controls consisted of the uninoculated medium extracted, concentrated and assayed in the same manner. This experiment was repeated twice.

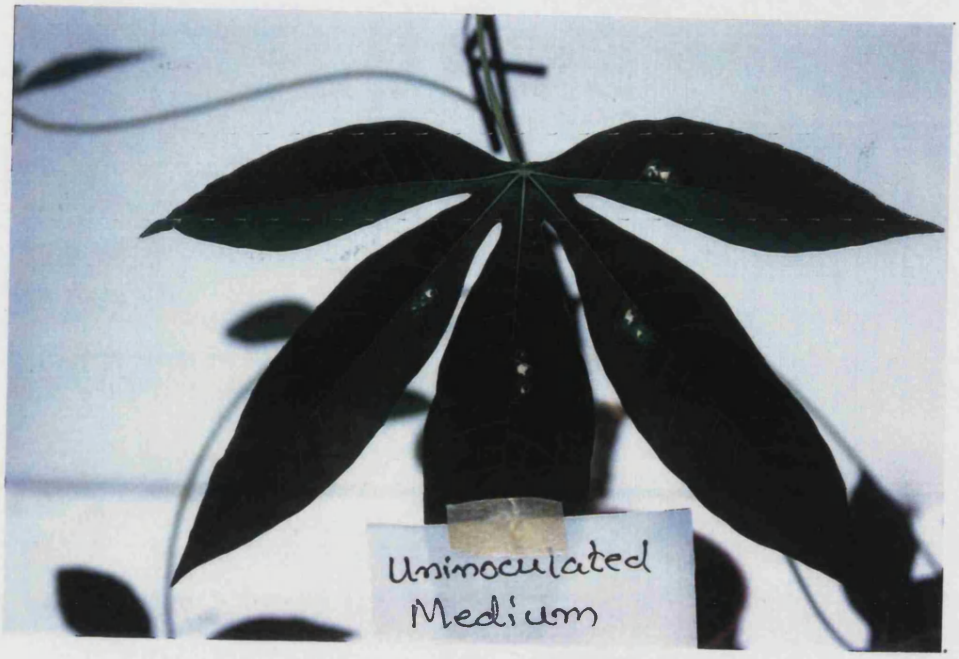
When unextracted, both uninoculated medium and culture fluids produced a bleached effect on leaves (Plates 13 and 14).

The extracts of both Xcm isolates 3194 and 2967 produced necrosis (which resembled a bleached effect rather than the brown discoloration caused by Xcm) in the infiltrated areas of the leaves within 24h (Plates 16, 17), but the necrosis did not spread beyond this area. The extracted uninoculated medium did not show any effect on the leaves (Plates 15 & 22). The dilutions of the extracted culture fluid tested yielded this effect but the degree of bleached effect decreased with dilution (Plates 18-20).

Plate 13- Leaf of cassava cultivar MCol 22 infiltrated with unextracted uninoculated Watanabe medium. Note the bleaching of leaf similar to that induced by fluids from bacterial cultures (see Plate 14).

Plate 14- Leaf of cassava cultivar MCol 22 infiltrated with unextracted culture fluid of Xcm isolate 2967 grown in Watanabe medium for 6 days.

13



14

Plate 15- Leaf of cassava cultivar MCol 22 infiltrated with uninoculated but extracted Watanabe medium. The extraction method of Perreaux et al. (1982) was followed. Note the absence of bleaching of leaves as seen with the unextracted medium.

Plate 16- Leaf of cassava cultivar MCol 22 infiltrated with extracted culture fluid of Xcm isolate 3194 grown in Watanabe medium for six days.

Plate 17- Leaf of cassava cultivar MCol 22 infiltrated with extracted culture fluid of Xcm isolate 2967 grown in Watanabe medium for six days.

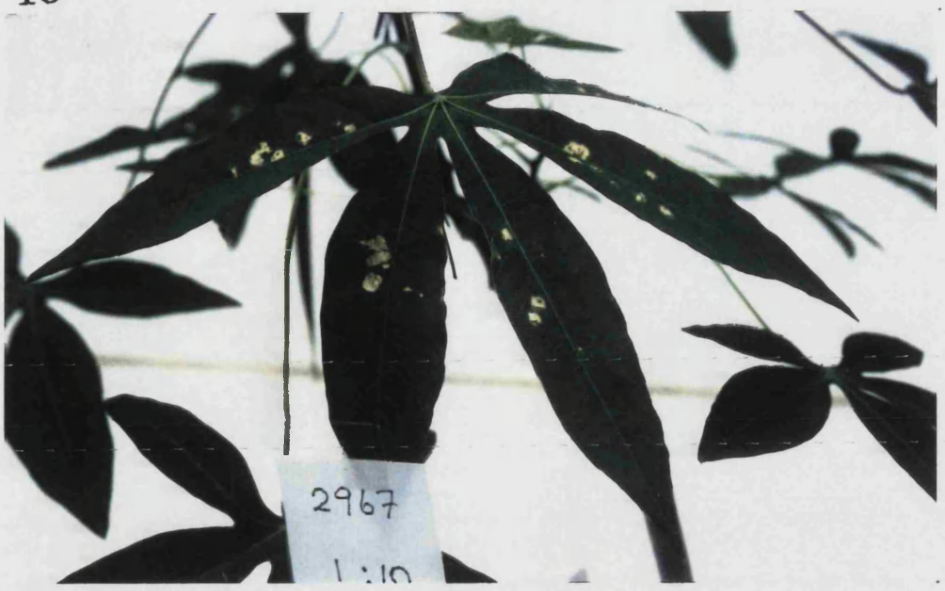
15

16

17

Plates 18-20- Leaves of cassava cultivar MCol 22 infiltrated with the above culture extract of Xcm isolate 2967 diluted x10, x100 and x1000.

18



19

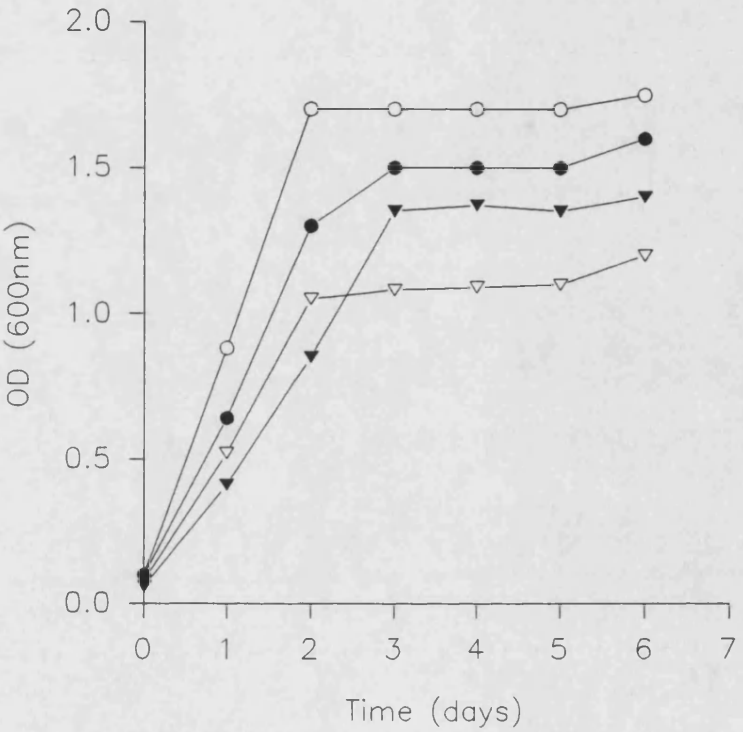
20

5.The effect of reduced levels of amino acids in Watanabe medium on growth and toxin production

Watanabe medium contains 0.5g/l methionine and 1g/l glutamic acid. The toxin described by Perreaux et al. (1982) is derived directly from methionine. A separate experiment showed that this medium supplemented with only 0.01g/l amino acids (glutamic acid, methionine, cysteine, leucine, isoleucine, phenylalanine and tryptophane) supported bacterial growth equivalent to that on full strength Watanabe medium. Moreover, these high levels of amino acids in Watanabe medium are unlikely to occur free in plants. Therefore, the toxicity of extracts when bacteria were grown in Watanabe medium with lower levels of amino acids were investigated.

Xcm isolate 2967 was grown in Watanabe medium supplemented with full strength methionine and glutamic acid and 1/10, 1/50 and 1/100 concentrations of these amino acids. The growth rates of the bacterium in these concentrations were monitored and Fig. 18 shows that although the most rapid growth was in the full strength medium, the final growth attained after 2 days was high even with 1/100 concentration.

Fig. 18– Growth of Xcm isolate 2967 in Watanabe medium with different concentrations of two amino acids



○ full strength methionine and glutamic acid
● 1/10 strength methionine and glutamic acid
▼ 1/50 strength methionine and glutamic acid
▽ 1/100 strength methionine and glutamic acid

Each point in the graph is the mean value of four replicate samples.

After 6 days the culture fluids were extracted with ethyl acetate and dried residue redissolved in SDW to give an effective x40 concentration using the same procedure as in the previous experiment. The crude toxin preparation was then filter sterilised, diluted x10, $x10^{-2}$ and $x10^{-3}$ and infiltrated into leaves of susceptible cultivar M Col 22.

The degree of necrosis produced by the extracts on leaves decreased with decreasing levels of amino acids in the medium. The leaves infiltrated with the extract from the cultures with complete levels of amino acids showed necrosis on leaves within 24 h. This again resembled a bleaching effect which was unlike the symptoms caused by Xcm and did not spread beyond the areas of infiltration. The extracts from cultures grown in 1/10 and 1/50 the amounts of amino acids resulted in only slight necrosis and that from cultures with 1/100 amino acid levels only gave faint brown markings along the areas of infiltration. (Plates 21, 23, 24, 25 & 26). This indicates that high amino acid levels are required for the production of a toxic metabolite(s) by the bacterium.

Plate 21- Leaves of cassava cultivar MCol 22 infiltrated with culture extracts of Xcm isolate 2967 grown in Watanabe medium with (from left) full strength, 1/10, 1/50 and 1/100 concentration of glutamic acid and methionine.

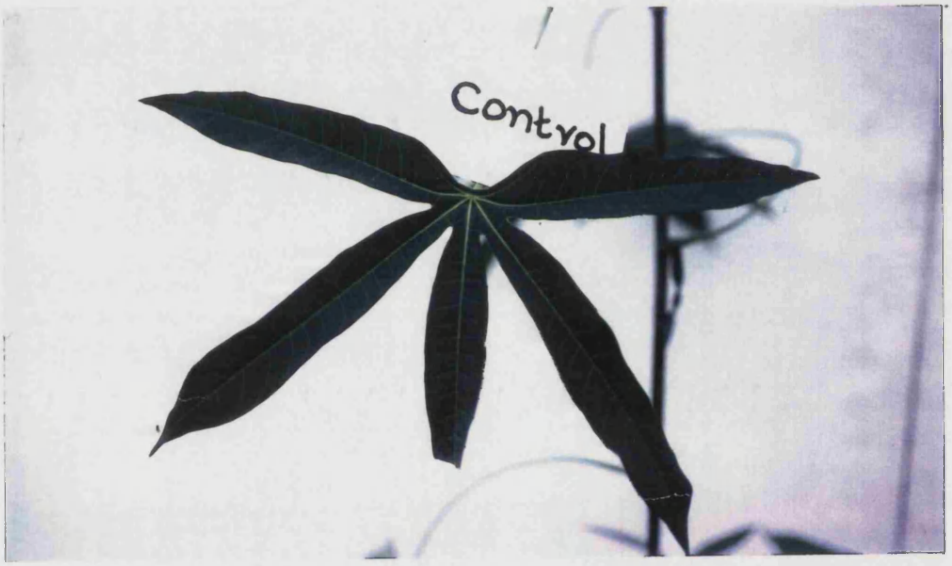
Plate 22- Leaf of cassava cultivar MCol 22 infiltrated with uninoculated extracted Watanabe medium with full strength amino acids.

Plates 23-26- Leaves of cassava cultivar MCol 22 infiltrated with culture extract of Xcm isolate 2967 grown in Watanabe medium with full strength, 1/10, 1/50 and 1/100 amino acids.

21


2967 in
Wat. med.
full strength
2767 in
1/2 a acids
1/2 w.m.
1:1
2967 in
1/2 a.acids
1/2 w.m.
1:1

22

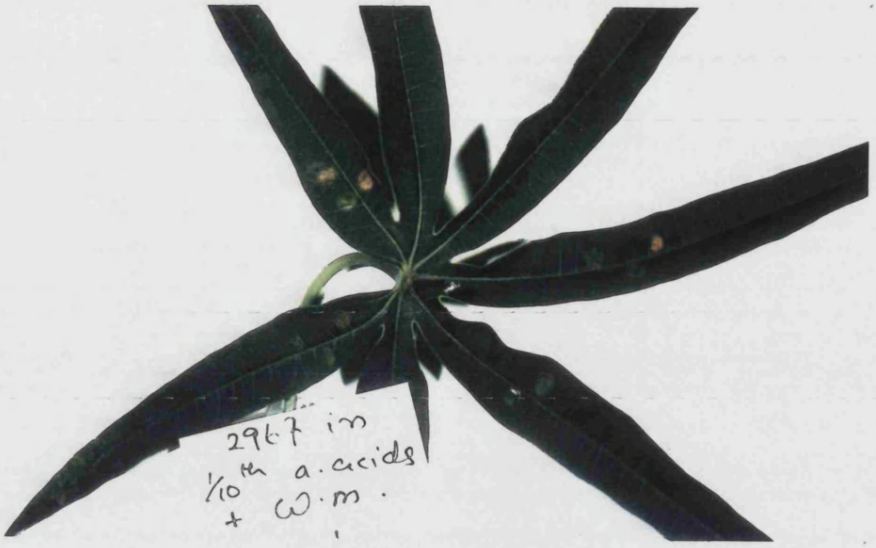

Control

23

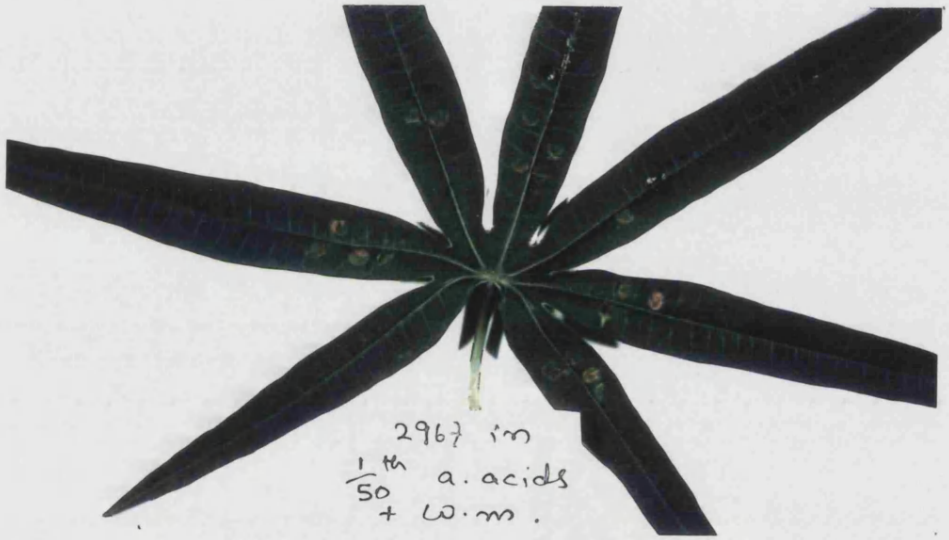

2967 in
Wat. med.
full strength

1/10, 1/50 and 1/100 amino acids.

24


2967 in
1/10th a. acids
+ w.m.

25


2967 in
1/50th a. acids
+ w.m.

26


2967 in
1/100th a. acids
+ w.m.

6.Amino acid analysis of cassava leaves

In view of high levels of methionine being a prerequisite for toxin production by Xcm, the levels of free and bound methionine were analysed in cassava leaves before and following infection. Investigation of methionine levels in lesions was deemed necessary in light of the modes of action of phaseolotoxin and tabtoxin which result in considerable accumulation of certain amino acids in diseased leaves (Gilchrist, 1983; Durbin, 1982).

Third and fourth leaves of 2 month old cassava plants were infiltrated with a suspension of Xcm isolate 2967 ($1x10^8$ cfu/ml) and samples were dissected out after 9days from (a)the lesions that developed, (b)the adjacent symptomless tissue, (2cm zone surrounding the lesion). This latter symptomless tissue contained high numbers of Xcm (Section II, 2ii), (c)leaves infiltrated in a similar manner with SDW and (d)untreated healthy leaves. Samples from infected and control leaves were instantly preserved in liquid N_2 and freeze dried for storage.

Table 25- Levels of methionine in Watanabe medium and leaf tissue

Free methionine levels in leaf tissue (mg/g)		Total methionine levels in leaf tissue (mg/g)	
++on dry wt. basis	on fresh wt. basis	++on dry wt. basis	on fresh wt. basis
1.* 1.2×10^{-5}	1.0×10^{-5}	2.72×10^{-2}	2.39×10^{-2}
2.* 2.0×10^{-4}	1.7×10^{-4}	2.80×10^{-2}	2.46×10^{-2}
3.* 1.2×10^{-4}	1.1×10^{-4}	2.48×10^{-2}	2.43×10^{-2}
4.* 4.0×10^{-5}	4.0×10^{-5}	2.84×10^{-2}	2.49×10^{-2}

1.* healthy tissue

2.* leaf tissue infiltrated with SDW

3.* leaf tissue from necrotic area

4.* leaf tissue from area surrounding (2cm on either side) necrotic lesion

++Dry wt. of samples was determined by lyophilyzation of fresh samples.

Methionine levels in Watanabe medium (mg/ml)-

full strength	1/10	1/50	1/100
0.5	0.05	0.01	0.005

Full data of the amino acid analysis is given in Appendix IV.

The analysis of free and total amino acids was done as described in Materials and Methods and Appendix III. This showed that the levels of free methionine was lower than that of all other amino acids and also that the levels in all leaf samples tested were considerably lower (x250-x25) than that in Watanabe medium (Table 25). There was no increase in methionine levels in diseased leaf samples above the levels in healthy and water infiltrated

leaves. However, the level of total methionine was approx. x135 higher then the levels of the free form in all leaf samples indicating availability for release by proteolytic enzymes. If the bound methionine were to be released by proteolytic activity, the levels (approx. 0.03mg/ml) would be sufficient for toxin production as culture extracts with methionine levels as low as 0.01mg/ml in Watanabe medium showed toxicity. When the toxicity of extracts of Xcm grown in Watanabe medium with decreasing levels of amino acids were tested in a previous experiment (Sectin III,5), it was evidenced that 0.005mg/ml levels of methionine in the medium was insufficient to produce the 'toxin'. However, the methionine levels found in all cassava leaf samples were x25 less than this.

Analysis of total amino acids also showed that the levels of all the amino acids sampled remained approximately the same. However, the levels of free amino acids varied considerably (Appendix IV). There was a general increase over healthy control leaf tissue in serine, threonine, glycine, alanine, valine, isoleucine, leucine tyrosine, phenylalanine, histidine, arginine and lysine in infected leaf tissue. The levels of aspartic acid and glutamic acid decreased in infected tissue and were minimal in the necrotic area. The levels of arginine and lysine were also the least in this area. There was a remarkable increase in proline levels in the

necrotic area.

7. Synthesis of protease by Xcm on various carbon and nitrogen sources

In view of the requirement for high methionine levels for toxin production and the very low levels of free methionine in leaves, the possibility of Xcm producing protease enzyme which could release bound methionine in cassava leaves was investigated.

All eight Xcm isolates were assayed for protease production on skimmed milk NYGA plates. Proteolytic activity was indicated by a clearing zone around bacterial colonies. All Xcm isolates tested except 3191 showed proteolytic activity in this plate assay (Plate 27).

Xcm isolates 2967 and 3172 were grown in basal medium with 0.5% glucose, cassava cell wall, casein or bovine serum albumin as sole sources of carbon in order to achieve conditions for possible induction or derepression of protease synthesis. When casein or bovine serum albumin was used the nitrogen source in the basal medium ($NH_4H_2PO_4$) was omitted. 100ml of each medium (pH 6.8) in 250ml flasks were inoculated with 10ml of a suspension of bacteria ($1x10^8$ cfu/ml) and the flasks were incubated in a rotary shaker (150rpm) at 29°C. Four replicate flasks for each medium with isolate 2967 were

sampled after 48 and 72h, and isolate 3172 after 72h for protease activity.

Extracellular protease production by both Xcm isolates was low (Table 26). No activity was detected when bacteria were grown on BSA or cell walls. There was some activity on casein but the highest activity was produced on the glucose.

Table 26- Protease activity of Xcm

N/C source	Assay pH							
	pH 5				pH 8			
	2967		3172		2967		3172	
	a	b	a	b	a	b	a	b
casein	0.04	0.05	-	0.03	0.19	0.14	-	0.07
BSA	0	0	-	0	0	0	-	0
glucose	0.31	0.21	-	0.23	0.34	0.46	-	0.08
cell wall	0	0	-	0	0	0	-	0

a- samples taken after 48h b- samples taken after 72h

Plate 27- Detection of protease activity of Xcm isolates on skimmed milk NYGA plates.
Proteolytic activity is indicated by a clearing zone around bacterial colonies.

27

B). Investigation of the involvement of a cell wall degrading enzyme in pathogenesis

The symptoms caused in plants by phytopathogenic Xanthomonas spp. are unlike those induced by the pectolytic soft rot Erwinia, Bacillus and Pseudomonas species (Starr & Nasuno, 1967). However, Burkholder & Starr (1948) found that about 14 Xanthomonas spp. could liquefy a pectate gel. Similar observations have been made by other workers (Sabet & Dowson, 1951; Smith, 1958; Dye, 1960; Ikotun,1984a & b; Abo-El-Dahab, 1964; Liao & Wells, 1987; Angeles-Ramos, 1991; Dow et al.,1987 & 1989; Beaulieu, 1991). Pectate lyase and pectin methyl esterase have been identified from xanthomonads (Starr & Nasuno, 1967; Dye, 1960; Smith, 1958; Dow et al., 1987,1989) including Xcm (Ikotun, 1984a). In view of these findings, the possible involvement of pectolytic cell wall degrading enzymes in the pathogenesis of cassava by Xcm was investigated.

Initially, enzyme activity was determined in culture fluids of Xcm grown on different carbon sources. This led to a detailed study of pectate lyase (PGL) in vitro and in vivo.

1. Synthesis of cell wall degrading enzymes in culture on cell walls

The cell wall of a host plant contains a range of

polysaccharides to which the spectrum of cell wall degrading enzymes (CWDE) of the pathogen would be expected to correspond and for this reason cell walls should be a suitable inducing substrate for the synthesis of such enzymes (Cooper & Wood, 1975). As the cell walls are mainly insoluble in the medium, they should not cause catabolite repression of enzyme synthesis.

Cell walls of cassava stems and petioles were used in cultures as the sole carbon source added at 0.5% to 100ml of minimal medium B (pH 6.8) in 250ml flasks. After inoculation with 10ml of four pathogenic Xcm isolates suspension ($1x10^8$ cfu/ml) the flasks were incubated in a rotary shaker (150rpm) at 29°C. Samples were removed after 5 days and assayed for pectate lyase (PGL), polygalacturonase (PG), pectin methyl esterase (PME) and pectin lyase (PL) activity and for bacterial growth (Materials and Methods 15a, b, c and d).

Table 27- Production of cell wall degrading enzymes by Xcm on host cell walls as sole carbon source

Isolate	PG(a)	PL(b)	PGL(c)	PME(d)	Bacterial growth (cfu/ml)
2444	0	0	0.07	0	$6.1x10^9$
2967	0	0	17.46	0	$5.0x10^9$
3172	0	0	0.09	0	$1.4x10^9$
3194	0	0	0.10	0	$3.7x10^9$

(a)PG activity assayed viscometrically (RVU)
(b)PL activity assayed by UV absorbance at 240nm (umol/ml/min)
(c)PGL activity assayed by UV absorbance at 235nm (umol/ml/min)
(d)PME activity assayed by titration (meq/ml/h)

Culture fluids obtained after 5days of growth and assayed after dialysis against DW (pH 7).

Table 27 shows that growth occurred on cassava cell walls but none of the Xcm isolates tested produced PL, PG or PME. However, high extracellular PGL activity was found with Xcm isolate 2967 but isolates 2444, 3172 and 3194 produced only low levels.

2.Growth and production of PGL by isolate 2967 on various carbon sources

Xcm isolate 2967 was grown on basal medium supplemented with 4 different carbon sources in order to understand regulation of enzyme synthesis. Cassava cell walls (0.5%) and sodium polypectate (NaPP)(0.25%) were used as potential inducers which should not effect

catabolite repression because of their insolubility or degree of polymerisation respectively; glucose was added (0.5%) to provide non inducing and repressing conditions and xylan (0.25%) as a non inducing, non repressing substrate; the latter two substrates would also reveal basal production, if present.

100ml of each medium (pH 6.8) in 250ml flasks were inoculated with 10ml of a suspension of Xcm isolate 2967 ($1x10^8$ cfu/ml) and the flasks were incubated in a rotary shaker (150rpm) at 29°C for 5 days. Samples were removed from flasks and bacterial growth in all media except on cell walls was monitored by measuring the OD at 600nm. Bacterial growth in the cell wall medium was monitored by dilution plate counts.

Fig 19 shows that there was an increase in bacterial numbers in all 4 media tested indicating an ability of the bacteria to degrade and utilise all carbon sources; however growth on xylan was poor.

Table 28 shows PGL activity in each culture supernatant after 3 and 5 days of bacterial growth. PGL synthesis after 5 days was slightly higher than that of after 3 days. PGL synthesis only occurred in cultures containing NaPP and cell walls as carbon sources. No basal synthesis was detected on glucose or on xylan. PGL levels produced were about 4 times higher on cell wall medium compared to the levels produced in the medium with

NaPP as the carbon source. It is therefore apparent that PGL production is controlled by induction and catabolite repression.

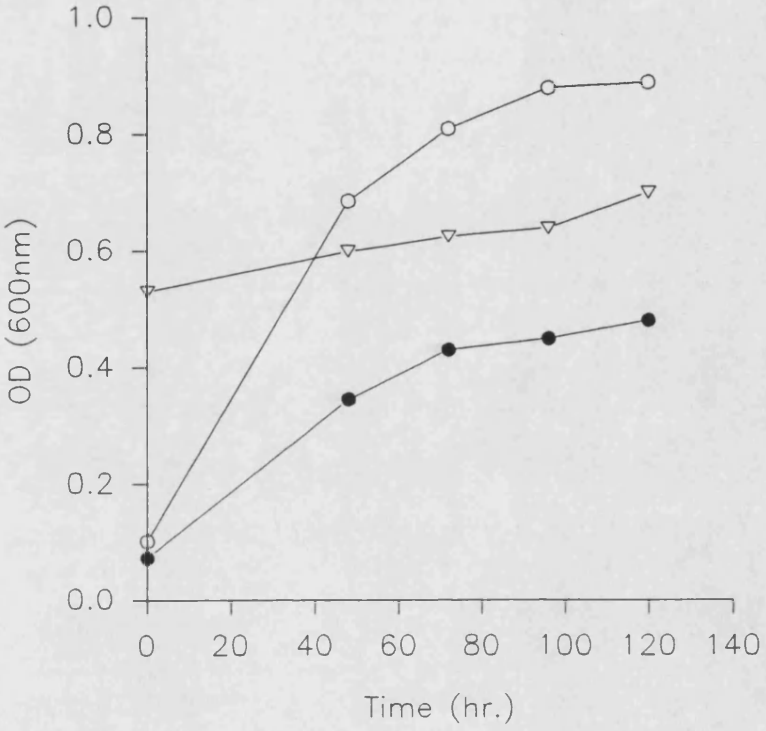
Table 28- Pectate lyase synthesis by Xcm isolate 2967 on various C sources

Day	PGL activity (umol/ml/min.)			
	glucose	xylan	NaPP	cell wall
3	0	0	2.46	8.06
5	0	0	3.35	11.01

PGL activity assayed by UV absorbance at 235nm.

Each reading is the mean value of three replicate assays

Fig. 19– Growth of Xcm isolate 2967 on four carbon sources



○ bacterial growth on glucose

▽ bacterial growth on xylan

● bacterial growth on NaPP

Growth of bacteria on cell wall medium

time(hr)	cell no.s (cfu/ml)
0	$1x10^7$
48	$1x10^8$
120	$1x10^9$

Resolution of Xcm PGL isozymes by isoelectric focussing

IEF was carried out to determine the total number of isozymes produced by Xcm. The isozyme complement would give some indication as to the number of structural genes that code for PGL in Xcm. Knowledge of pI and the number of isozyme forms would also assist future work on purification and possible molecular genetic analysis.

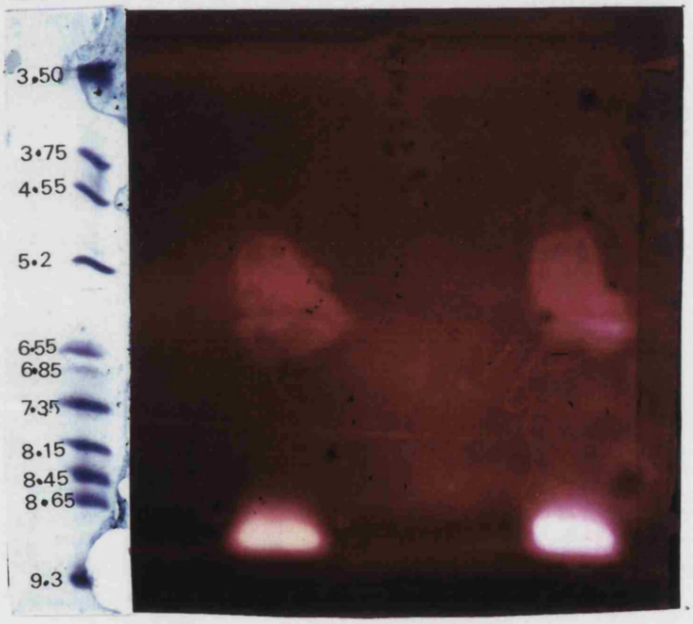
Broad range (pH 3-10) IEF of PGL (Ried & Collmer, 1984; 1985)

Dialysed filtrates of (i)Xcm isolates grown on cell wall medium and (ii)extracts from inoculated cassava leaves were concentrated against PEG (culture fluids 10 times and leaf extracts such that 1ml corresponded to 1g fresh wt. of leaf tissue respectively) and subjected to IEF on a Multiphor apparatus (LKB) for 11/2h (Materials & Methods Section 18a). Pectate agarose overlays (Materials & Methods Section 18b) were placed directly onto the gel and maintained at 30°C for 5h. The overlays were then immersed in 0.05% Ruthenium Red for 20 mins and rinsed in DW and observed for cleared bands indicating the presence of PGL. This experiment was repeated three times with culture fluids of Xcm isolate 2967.

Culture supernatants of Xcm isolate 2967 showed a single distinct band in the region of pH 9 (Plate 28). Isolates 2444, 3172 and 3194 and also the extracts from infected cassava leaves failed to show any activity.

Plate 28- IEF profile of extracellular PGL produced by Xcm isolate 2967. A single isozyme is indicated by the white clearing zone on the red overlay. (pI~9.0).
Note glycine added to dialysed enzyme samples to overcome the deleterious effect of salts resulted in a slight clearing around its pI~5.8. This is probably due to complexing with PGL.

28



3.Maceration and cell killing ability of pectate lyase

There are many examples where PGL produced by bacterial plant pathogens have been shown to kill plant cells in association with maceration (Basham & Bateman, 1975a,1975b; Collmer et al., 1985; Liao, 1989). In view of this, the effect of PGL from Xcm isolate 2967 on cassava stem tissues and suspension cultured cells was investigated. Pure enzyme was not available but manipulation of culture conditions resulted in comparable culture fluids but with either high (on host cell walls) or no (on glucose) PGL activity.

Cassava stem discs were prepared as described in Materials and Methods 11b and placed in the following reaction mixtures-

Mixture 1- phosphate buffer (pH 6.5) 5ml
SDW 5ml
1mM $CaCl_2$

Mixture 2- phosphate buffer (pH 6.5) 5ml
dialysed supernatant of bacterial shake culture with glucose added as carbon source 5ml
1mM $CaCl_2$

Mixture 3- phosphate buffer (pH 6.5) 5ml
dialysed supernatant of bacterial shake culture with cell wall added as carbon source 5ml
1mM $CaCl_2$

Mixtures 1 and 2 served as controls (no PGL was detected in glucose cultures). The reactions were carried out at room temperature.

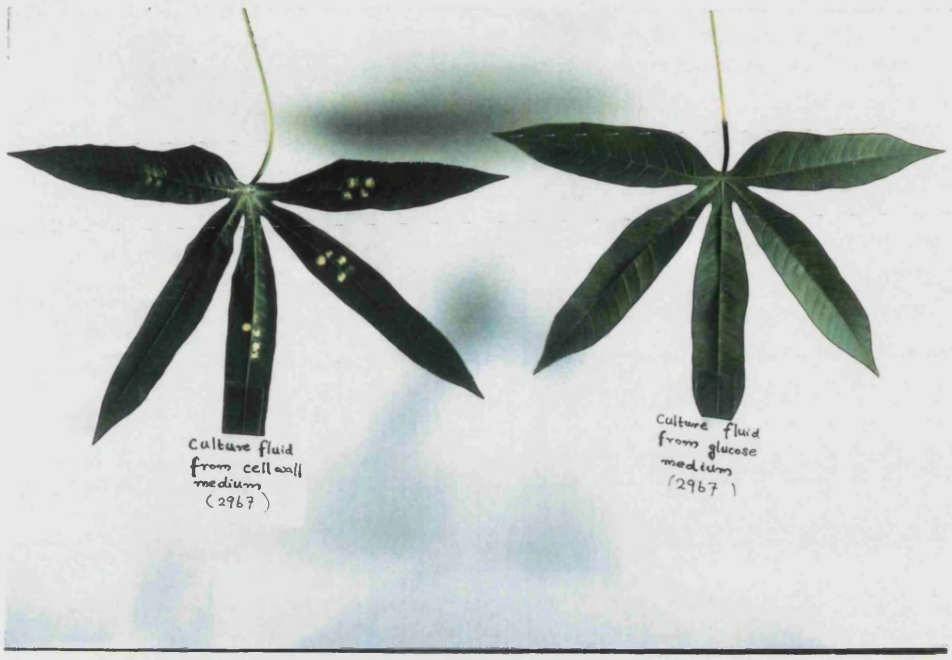
Cell wall culture supernatants started cell killing by 3h and continued the effect until almost all the cells in stem discs were killed after 10h. This correlated with tissue maceration which was initiated after 3h and increased until the coherence was completely lost after ca. 10h (Table 29). No toxicity or change in tissue integrity occurred with culture fluids lacking PGL.

The cell free dialysed culture fluids of isolate 2967 grown in cell wall and glucose media were also infiltrated into cassava leaves. The cell wall fluid produced browning on the leaves whereas the glucose medium failed to show any effect (Plates 29 and 30).

Plate 29- Leaves of cassava cultivar MCol 22 infiltrated with culture fluid of Xcm isolate 2967 grown in cassava cell wall and glucose media.
Note that respective activities of PGL were
11.01- 0umol/ml/min.

Plate 30- Detail of leaf of cassava cultivar MCol 22 infiltrated with culture fluid of Xcm isolate 2967 grown in cassava cell wall medium.

29


Culture fluid
from cell wall
medium
(2967)
Culture fluid
from glucose
medium
(2967)

30


Cult fluid
fro ll wall
med
(2)

Table 29- Effect of culture supernatants on cassava stem tissue

time (h)	reaction mixture	NRI	MI
1	1	0	0
	2	0	0
	3	0	0
3	1	0	0
	2	0	0
	3	1	0.5
6	1	0.5	0
	2	0.5	0
	3	2.5	2.5
8	1	1	0
	2	1	0
	3	3	3.5
10	1	1	0
	2	1	0
	3	4.5	5

The cell killing of stem discs was estimated by Neutral Red Index (Tribe, 1955). 5 discs were removed at each sampling time from each reaction mixture.

Maceration was tested by subjecting the discs to a standard stress between two dissecting needles (Maceration Index)

4. Effect of culture supernatants on suspension cultured cells of cassava

20day old suspension cultured cells of cassava cultivar MCol 113 were suspended at 1% v/v concentration

in the following reaction mixtures:

i)dialysed supernatants of bacteria grown in medium with glucose as carbon source

ii)dialysed supernatants of bacteria grown in medium with cell walls as carbon source (a dilution series of 1/10, 1/100, 1/1000)

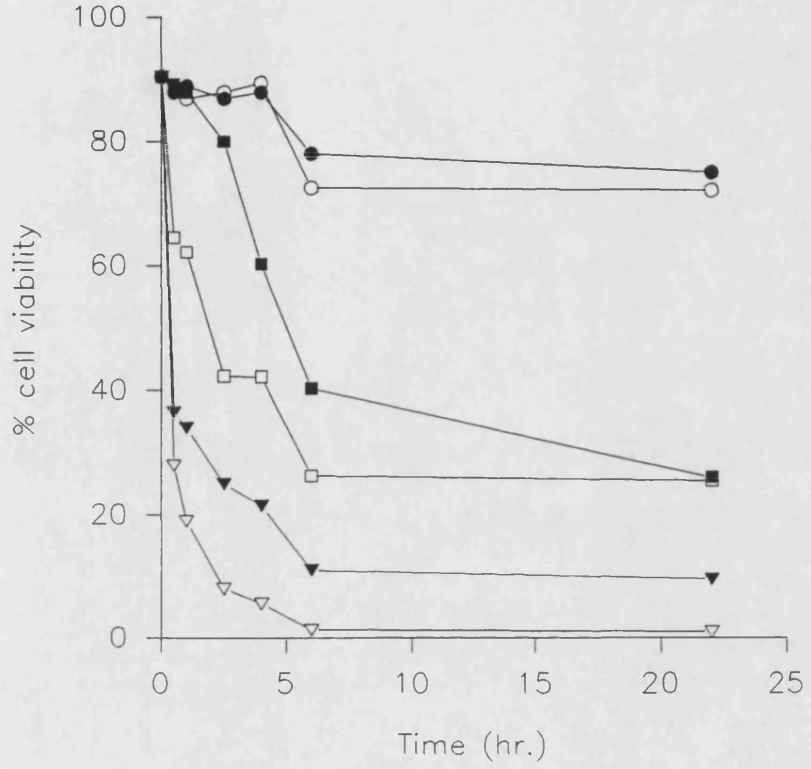
iii)above supernatant autoclaved to inactivate PGL

The viability of cells was monitored by staining with FDA.

Fig 20 shows that the autoclaved cell wall culture fluid and glucose culture supernatant had no effect on cell viability of suspension cultured cassava cells. In contrast the undiluted cell wall culture fluid caused ca 70% cell mortality by 30min which increased to ca. 98% by 6h. The ability to cause cell death decreased with the dilution of the supernatant but even at 1/1000 dilution ca. 75% loss of viability occurred by 22h.

In summary, these data indicate that Xcm produces an inducible PGL capable of killing and macerating cassava tissue.

Fig. 20– Host cell killing by pectate lyase



PGL activity of undiluted culture fluid 15.80 umol/ml/min

Suspension cultured cassava cells were suspended in:

- ○ Control (culture fluids from bacteria grown in glucose)
- ■ 1/1000 dilution of the supernatant from Xcm cultures with host cell walls
- □ 1/100 dilution of the supernatant
- ▼ 1/10 dilution of the supernatant
- ▽ undiluted supernatant
- ● heat treated supernatant

5.Extraction of PGL from inoculated cassava tissue

The previous findings show that PGL is a potential determinant of pathogenicity of Xcm; therefore attempts were made to detect the enzyme during infection.

Leaves of 2month old susceptible cassava plants were infiltrated with a suspension of Xcm isolate 2967 ($1x10^8$ cfu/ml). The lesions that developed around the area of infiltration and symptomless but Xcm colonised leaf tissue (Section II, 2ii) from a 2cm zone surrounding lesions were removed after 9 days for extraction of enzymes. Controls consisted of tissue from leaves infiltrated with SDW. Extractions were done as described in Materials and Methods 18 and assayed for PGL using the 235 assay. This experiment was repeated twice.

Pectate lyase activity was not detected in any of the leaf tissue sampled. Similarly IEF activity staining (see above) did not reveal PGL activity of these extracts.

DISCUSSION III

In order to investigate the possible involvement of a toxin in pathogenesis, the initial strategy was to employ various culture conditions with a view of finding the optimum for synthesis of toxin(s) by Xcm. The culture fluids of two Xcm isolates grown in Minimal medium B failed to show toxicity in any of the bioassays used. The different bioassays used in this study are likely to vary in their sensitivity as they employ different reponses of different cell types which is a desired approach in the early work on a putative toxin (Cooper, 1993). Assay of symptoms is commonly used but can be slow, ambiguous and the least sensitive. An example of the varying sensitivity of bioassays is the responses in maize to various dilutions of cultures containing HMT-toxin i.e. lesion formation 10:1, electrolyte loss 1:50, inhibition of photosynthesis 1:200, inhibition of root growth 1:3000, inhibition of dark CO_2 fixation 1:10000, loss of chlorophyll 1:50000 (Wheeler, 1976). In the present study, measurement of K^+ leakage from treated cassava stem and leaf discs and the mortality of suspension cultured cassava cells treated with the culture fluids might be expected to be more sensitive than induction of symptoms in leaf infiltration.

The culture fluids were obtained after early and late log phases of growth (36 and 48h) as production of

toxins is reported to occur during different phases of growth such as phaseolotoxin and tabtoxin in early log phase, AM toxin in mid-log phase, syringomycin in stationary phase and lycomarasmin during autolysis (Cooper, 1993). The culture fluids obtained at either growth stage in the present study did not show any toxicity.

Restricted supply of C, N and S provides a more natural environment and affects metabolism by relieving catabolite repression of synthesis of many catabolic enzymes (Cooper, 1974) and this could be true for toxin synthesis too but such effects have rarely been studied for toxins. In an attempt to supply nutrients in a restricted manner, they were added in diffusion capsules and allowed to diffuse slowly into the medium to prevent their accumulation and their interference in toxin bioassay. A problem with the use of culture fluids with high salt levels is the possible toxicity induced by them following concentration of the fluids (eg. Buchanan & Starr, 1980). However, the culture fluids of Xcm isolates 2967 and 3194 grown under these restricted conditions also failed to show any toxicity by any of the bioassays.

When Xcm isolates 2967 and 3194 were grown in Watanabe medium and extracted following the method used by Perreaux et al. (1982), toxicity towards cassava

leaves was demonstrated, although the bleaching of leaf tissue did not resemble disease symptoms. Also, Watanabe medium contains 0.5g/l methionine and 1g/l glutamic acid. When the levels of these amino acids were reduced, the levels of toxicity of the extracts decreased as well such that no toxicity was detectable ≤0.005mg/ml. This indicates that high amino acid levels are required for the production of a toxic metabolite by the bacterium but only 10µg/ml was required for Xcm growth (Section II,1,ii). Analysis of methionine levels in planta showed that it was between $1.7x10^{-4}$-$1.0x10^{-5}$mg/g fresh weight This is lower than even the levels ($5.0x10^{-3}$mg/ml) in 1/100 diluted Watanabe medium. Therefore it could be assumed that the methionine levels present in cassava leaves are insufficient for toxin production although sufficient for bacterial growth.

Perreaux et al. (1982) extracted a toxin, 3 methyl thiopropionic acid (MTPA), from culture fluids of Xcm grown in Watanabe medium and this was shown to cause necrosis in cassava leaves. MTPA was later shown to be catabolized from methionine via transamination and subsequent decarboxylation of the intermediate ✂-keto acid (Ewbank & Maraite, 1990). Robeson & Cook (1984) showed that when X. campestris pv campestris was grown in Watanabe medium, trans-3-methylthioacrylic acid and 3-methylthiopropionic acid were produced. However, they

also showed that when the amount of methionine supplied to cultures was increased or decreased above or below that of Watanabe broth, a corresponding change in levels of the acids resulted. Neither of the acids was detected in cultures when methionine was omitted from the medium. The results of the present study indicated a similar effect of toxicity of the culture extracts. Similarly, with regard to the precursor for MTPA and MTAA Chiang & Nip (1973) reported zero levels of free methionine in leaf tissue of three cabbage cultivars tested. Robeson & Cook (1984) also suggested that the activity of the above acids may be solely due to their acidic nature as the phytotoxicity was completely eliminated in a buffered solution at pH 6.5. MTPA was also shown to be present in artificially and naturally infected cassava leaves using gas liquid chromatography (Perreaux et al., 1986). It could be argued that its presence does not mean that it has any significance in pathogenicity or even virulence but it does suggest that the necessary conditions for the production of the toxin are available in the plant. These conditions must be obtained by the bacterium changing the composition of plant cells in such a way that the intercellular environment is conducive for toxin production by Xcm. One mechanism might be that Xcm could produce proteolytic enzymes to break down proteins rapidly so that the levels of methionine increase to the levels required for toxin production.

Patel and Walker (1963) reported that susceptible plants of beans infected with Pseudomonas syringae pv phaseolicola showed a pronounced increase of many amides and amino acids such as ornithine, histidine, methionine, asparagine, glutamine, β alanine and lysine. Although this was proved otherwise later (Gilchrist, 1983), they suggested at the time that this increase in amino acids in diseased tissue was due to breakdown of leaf proteins by proteolytic enzymes produced by the pathogen.

Many microorganisms produce proteases in culture (Davies, 1963; Hagihara, 1960) but only a few are known to degrade native proteins. Keen et al. (1967) showed that Pseudomonas lachrymans produced caseinolytic protease in culture and during pathogenesis in cucumber. Protease activity on gelatin was obtained from several spp. of Pseudomonas (Hagihara, 1960) and Friedman (1962) postulated that virulence of Erwinia carotovora could be attributed to increased proteolytic activity.

A study on the production of protease by Xcm showed that the bacterium produced low extracellular activity in basal medium with glucose or casein added as the carbon source but not in BSA or cassava cell walls. This unusual pattern of production does not concur with the ususal regulation of microbial protease by carbon and/or nitrogen derepression which may or may not also involve induction by proteins (St.Leger et al.,1988; Cohen, 1981;

Cohen & Drucker, 1977). Protease activity was also shown by all eight Xcm isolates on an assay on agar plates. Protease production by Xcm means that in theory the bacterium could degrade leaf proteins to increase methionine to levels sufficient for toxin production. However, an analysis of free and total amino acid levels in inoculated, water infiltrated and untreated cassava leaves showed that there was no significant difference in methionine content of leaves given the different treatments. However, if total methionine in leaves was released by the proteolytic activity of the bacterium the resulting levels should be sufficient for toxin production. Amino acid analysis of healthy and infected cassava leaf tissues was carried out only once and therefore should be repeated for these results to be conclusive.

The crucifer pathogen X. campestris pv campestris is reported to produce extracellular enzymes including amylase, endoglucanase, polygalacturonate lyase and protease; but analysis of protease minus mutants showed that extracellular protease is not critically important in pathogenicity of X. campestris but may play a minor role in disease development (Tang et al., 1987). However, contrary to this, Dow et al. (1990) showed that a protease-deficient mutant of Xanthomonas campestris pv campestris showed considerable loss of virulence in

pathogenicity tests on mature turnip leaves. Reddy et al. (1971) also found protease activity in extracts from susceptible alfalfa plants infected with X. alfalfae and similarly Srivastava & Prasad (1989) found an intensive proteolytic enzyme activity in susceptible bean leaf tissues infected with Xanthomonas campestris pv phaseoli.

Alternatively, Xcm could block a metabolic pathway to result in accumulation of high levels of methionine to facilitate the production of the toxin/s. The clear example of this is phaseolotoxin produced by Pseudomonas syringae pv phaseolicola which inhibits the enzyme ornithine carbomyl transferase in host tissues, and thereby affects interlinked metabolic processes and results in the accumulation of certain amino acids (Gilchrist, 1983). Increases in the total amino acid content in infected plant tissue have been demonstrated in some plant diseases (Barnett & Naylor, 1966; Diener, 1960; Feldman & Hanks, 1964; Shaw & Colotelo, 1961). Singh & Smalley (1969) reported that there was an accumulation of proline, alanine and β - amino-n-butyric acid in the sap of elms inoculated with Ceratocystis ulmi. Similar general increases have been reported in peach leaves infected with Western X-virus (Diener, 1960), stem rust infected wheat (Shaw & Colotelo, 1961) and grape fruit seedlings infested with the nematode Radopholus similis (Hanks & Feldman, 1966) but this was not observed in the present study.

Even if a toxin is produced as a result of increased amino acid levels in the plant, the toxin must be considered as a virulence factor rather than a pathogenicity factor because its production would depend on other activities of Xcm which have already allowed the invasion of the host by the bacterium.

It could be argued that even if the methionine levels were increased by the pathogen these would not be detected if the amino acid was rapidly metabolized by Xcm. However, this possibility would seem unlikely as the leaf tissue in advance of symptom production also did not contain high levels of amino acids. These apparently healthy leaf areas contained high numbers of the pathogen and therefore even if the toxin was not produced at this stage the pathogen could have initiated the process that resulted in increasing the levels of the precursor.

In summary, it was not possible to show the synthesis of a toxin by Xcm under a range of in vitro conditions and the conditions necessary for the synthesis of a previously reported toxic metabolite seemed unavailable in cassava plants.

The possible involvement of pectolytic cell wall degrading enzymes in pathogenesis by Xcm was also investigated. The conditions required for enzyme

synthesis *in vitro*, activity *in vivo* and the effect of cell wall degrading enzymes on cassava tissues were examined.

Supernatants from *Xcm* cultures containing cassava cell walls showed no activity of PL, PME or PG. The work by Dye (1960) and Ikotun (1975) also showed that there was no production of PME by *Xanthomonas*. However, later observations by Fowle & Ikotun (1978) and Ikotun (1984) proved the production of an inducible PME by *Xcm*. Starr & Nasuno (1967) reported pectinesterase and polygalacturonic acid trans-eliminase (syn. pectate lyase or PGL) production by *Xanthomonas* spp. *X. campestris pv campestris* also produces a number of extracellular enzymes including PGL (Daniels *et al*., 1984; Nasuno & Starr, 1967).

The only significant activity detected from *Xcm* was pectate lyase (PGL) and for isolate 2967 this was produced mainly when polygalacturonan (as sodium polypectate) or cassava cell walls was supplied as the carbon source; levels achieved on cell wall medium were considerably (approx. tenfold) higher.

Regulation of PGL production by *Xcm* appears to follow production of most polysaccharidases by bacteria and fungi which are under the dual control of induction and catabolite repression (Magasanik, 1961; Cooper,

1977). Production of an inducible CWDE depends on the presence of a potential inducer that is usually derived from the polymeric substrate on which that particular enzyme is active. Polygalacturonan stimulates production of pectic enzymes of a wide range of bacteria and fungi. For some fungi the inducer appears to be monogalacturonic acid (Cooper & Wood, 1975) whereas the unsaturated dimer induces the extracellular PGL of Erwinia chrysanthemi and Erwinia carotovora (Collmer et al, 1982). The production of inducible PGL by Xcm on cell wall medium indirectly shows the amenability of the pectic component in cell walls to degradation by this pathogen. There was no PGL activity in xylan or on glucose media to indicate basal synthesis or control by derepression alone; it is difficult to explain how induced synthesis is triggered in the absence of basal enzyme which is considered to effect the initial release of inducers from polymeric substrates (Cooper, 1977). Catabolite repression of polysaccharidases is well established especially for cellulases for which maximum levels attained in cultures are related inversely to accessibility of cellulose substrates to enzyme hydrolysis (Horton & Keen, 1966; Mandels & Reese, 1960). The higher levels of PGL produced by Xcm isolate 2967 on cell walls cf. pectate is likely to be a reflection of this effect.

Abo-El-Dahab (1964) showed that pectic enzyme formation by X. malvacearum increased significantly on

basal medium enriched with 1% pectin, 1% calcium pectate or 0.05% galacturonic acid. Liao & Wells (1987) reported a X. campestris isolate that produced PGL, PME and PG in media containing polygalacturonic acid or pectin but not in a medium containing glucose. Lyase induction by pectic substances has also been observed in Erwinia spp and in some strains of Pseudomonas marginalis and P. fluorescens (Bateman, 1968; Fuchs, 1965; Lange & Knosel, 1970; Smith, 1958; Zucker & Hankin, 1970; Zucker et al., 1972). Starr & Nasuno (1966) found that most pectic enzymes produced by phytopathogenic bacteria are inducible including PME production by 8 strains of Xanthomonas campestris.

Specific induction and catabolite repression of CWDE may be important in the movement of Xcm in xylem of cassava stems into which they were shown to be mainly confined (Section IV). Nutrients, especially carbon sources are low in xylem fluids (Dimond, 1972; Wood,1961), therefore catabolite repression is likely to be minimal. In the xylem, the available substrates apart from vascular fluids are the secondary cell walls of xylem vessels and middle lamella/ primary wall complex of pit membranes. Little is known about their role as sources of inducers but occasional formation of gels in vessels during infection could indicate the involvement of pectic enzymes.

Isoelectric focusing of the crude culture

Isoelectric focusing of the crude culture supernatant of isolate 2967 showed the presence of a single isozyme with a pI of ≥ 9. The culture fluids of other isolates and the extracts from plants failed to cause any clearing zones in the overlay which reflects presumably the low PGL production by other isolates and in plant extracts according to the highly sensitive 235 assay.

Coincidentally, pectolytic strains of X. campestris pv vesicatoria produced a single pectate lyase with a pI of 8.8 (Beaulieu et al., 1991). Analysis of Pseudomonas viridiflava has also shown a single PL with a pI of 9.7 and for the two PGL's of P. fluorescens the major isozyme has a pI of 10 and the minor form a pI of 6.7 (Liao, 1989). Erwinia spp. have been shown to have more than one PGL by various workers (Liao, 1989; Ried & Collmer, 1985; Boccara et al., 1988; Collmer et al., 1985; George et al., 1991). Extracellular PGL from Erwinia carotovora subsp. atroseptica was shown to have six endo PGLs with pIs of 10.2, 9.6, 9.5, 9.4, 9.2 and 8.9 (George et al., 1991) and E. chrysanthemi five PGLs with pIs ranging from 4.5 to 9.2 (Bertheau et al., 1984; Pupillo et al., 1976).

Dialysed, cell-free, cell wall culture fluids caused death of cassava stem cells, suspension cultured cells and resulted in maceration of cassava stem tissues; this only occurred with culture fluids containing PGL. Thus it

can be presumed that the cleavage of the α1-4 bonds in the rhamnogalacturonan in the cell wall by PGL in the culture fluid results in a loss in the ability of the pectic matrix of the wall to maintain the coherence of the wall. The maceration of cassava tissue by the culture supernatant indicates the activity of an endo type of enzyme (Basham & Bateman, 1975a). The cell wall breakdown may be responsible for cell death as both phenomena occurred concurrently in cassava stem discs. The membranes of these cells may be damaged either by a chemical interaction with the byproducts of enzyme degraded cell wall or by degradation of pectic components of cell wall thus weakening it so that the balance of the turgor pressure exerted by the protoplasts can no longer be borne (Bateman, 1976; Hall & Wood, 1970). This latter effect is suggested by experiments with pectate lyase from Erwinia chrysanthemi and potato tissue (Basham & Bateman, 1975b). However, Keon (1985) showed that cell wall degradation appeared to accompany rather than precede injury to cells and that the gross ultrastructural integrity of the cell walls appeared little changed during the early stages of injury.

Observations of cell mortality of suspension cultured cassava cells treated with different concentrations of the culture supernatant showed a graded killing response that correlated positively with the concentration of the supernatant. Levels as low as 1/1000

concentration of culture fluids (PGL activity=0.017 umol/ml/min) resulted in ca. 75% cell mortality. Cultured apple cells exposed to purified PL from Monilinia fructigena have also been reported to show a similar graded response to different concentrations of the enzyme (Hislop et al., 1979). An ultrastructural study of enzyme treated cassava cells would allow a deeper understanding of the nature and extent of damage caused in each instance.

Other Xcm isolates tested showed relatively low levels of PGL production in cell wall medium but these could be sufficient for pathogenesis in view of the cell killing by trace PGL activity from isolate 2967. Indeed the amounts produced by the isolates other than 2967 were higher than this.

PGL activity was not detected in extracts derived from either established or spreading lesions from inoculated plants. This could indicate the absence of enzyme production in vivo or the inactivation of the enzymes by a plant component (eg. phenolics) during infection or between the time of extraction and assaying. Ultrastructural studies did not show extensive cell wall damage (Section IV) and thus did not indicate an obvious role for pectic enzymes in pathogenesis. This was in contrast to the ultrastructural work by Ikotun (1975) who reported massive wall breakdown of cassava caused by

Xcm. However, only low levels of enzyme could be important for cell wall weakening sufficient for initial entry into xylem tissues and also for subsequent movement especially through pectic-rich pit membranes at vessel ends (Sections II & IV).

Non pathogenic mutants of X. campestris pv campestris have been obtained which had a PGL activity comparable to the wild type but showed ca. 3 fold lower extracellular activity and higher intracellular activity than the wild type. A plasmid PIJ 3000, which restored pathogenicity of mutants to turnip seedlings also restored PGL distribution to near that of the wild type (Dow et al., 1987). Thus, it would indicate a role for PGL in pathogenicity of the above bacterium. However, work by Dow et al. (1989) showed that mutants of X. campestris pv campestris specifically lacking isozyme 1 of PGL constructed by marker exchange of negative Tn5 insertions were as virulent as the wild type in pathogenicity tests on mature leaves or seedlings of turnip. These results suggested that isozyme 1 of PGL was not absolutely necessary for black rot pathogenesis suggesting a redundancy amongst the three major isozymes or an alternative role eg. one of the five PGLs of E. chrysanthemi determines systemic invasion of the host (Andro et al., 1984).

Similarly, biochemical and genetic analysis of

pectate lyase genes from X. c. pv vesicatoria have been carried out by studing mutants deficient in synthesis of PGL. Mutants were not altered in their ability to evoke disease symptoms, to grow in plants, or in their ability to induce HR in non hosts thus indicating that the pectate lyase gene had no essential role in pathogenicity of this pathovar (Beaulieu et al., 1991).

It can be concluded that although the synthesis of a PGL by Xcm could be demonstrated in vitro, the absolute necessity of this enzyme for pathogenesis of cassava by Xcm was not evident from TEM as extensive wall damage was not observed. The low production of PGL by some of the virulent Xcm isolates and the failure to detect the presence of PGL in vivo also seemed to indicate that PGL was not essential for pathogenesis. However, very low levels of the enzyme proved to have a toxic activity towards cassava tissues and PGL was the only factor identified from Xcm capable of damaging host cells. In view of these findings, it is justified to pursue a molecular genetic approach. Mutants without PGL activity would provide a better understanding of the role of the enzyme. As the presence of only one form of PGL was indicated, it should render fairly simple molecular analysis as only the genes coding for a single form would have to be inactivated in contrast to the lengthy studies on Erwinia spp. where 4-6 forms have had to be considered

(Collmer et al., 1985; van Gijsegem et al., 1985; Boccara et al., 1988; Roeder & Collmer, 1985; George et al., 1991).

Tests on the purified enzyme are also required for the results to be conclusive, but otherwise comparable glucose grown cultures which contain no PGL activity should indicate the presence of any other toxic components if present. To this end the very high pI value of the isozyme would facilitate purification by ion exchange chromatography.

Section IV - ULTRASTRUCTURAL STUDIES OF SUSCEPTIBLE CASSAVA TISSUES INFECTED WITH Xcm

Ultrastructural studies were carried-out using transmission electron microscopy (TEM) with the aim of gaining an insight into modes of infection, spread and tissue damage caused by Xcm in susceptible cassava stem and leaf tissues. In particular the questions to be answered concerned evidence for vascular or intercellular movement, toxin activity and cell wall degradation.

Two month old plants of susceptible cassava cultivar MCol 22 were used for all ultrastructural studies. All inoculations were carried out with a suspension of Xcm isolate 2967 ($1x10^8$ cfu/ml). The control plants were treated with SDW in a similar manner.

The mid lobe of third and fourth leaves were inoculated by leaf infiltration (Materials and Methods 9). Samples for microscopy were taken from the outer edge of necrotic areas that developed after 6 days. This area was faintly chlorotic and a separate study of bacterial multiplication showed the bacterial numbers in these leaf tissues were ca. 10^5 cfu/sq.cm by 6 days (Section II).

Stems of plants were inoculated at the fourth leaf axil by stem injection (Materials and Methods 9). Tissue for TEM was obtained 12 days after inoculation from 3cm above the point of inoculation. At this stage symptoms of

water stress were apparent in the petiole at the point of inoculation. A separate qualitative study of spread of Xcm in cassava plants inoculated in the same manner demonstrated the presence of bacterial cells 3cm above and below the point of inoculation by 5h (Section II). For light microscopy, free hand sections were obtained after 20 days, stained with ruthenium red and observed under a Olympus BH2 light microscope.

1.Uninoculated tissue

Bacterial cells were not observed in sections of uninoculated stem and leaf control tissue. The potential pathways for bacterial migration were evident in leaves as intercellular spaces between mesophyll cells and also between xylem cells (Plate 32C) and between chlorenchyma cells and xylem parenchyma cells in the vascular tissue in stems (Plate 34A & 34B) and xylem vessels. Xylem vessels range from several contiguous vessels in stems interconnected by pits to single vessels of leaflet veins (Plates 34C & 34E). Stomates could be likely ports of entry into leaves for the pathogen (Plate 32A).

2.Inoculated tissue

2.1.Vascular invasion

In both infected stems and leaves, bacteria were present in xylem tissue mostly concentrated in the lumen

of xylem vessels (Plates 35A-35N). Xylem parenchyma cells were also invaded but the point(s) of entry was not revealed. The xylem parenchyma cells containing bacteria were moribund with occasional traces of remaining organelles (Plates 35B, 35C, 35M). Surprisingly, in leaf tissue which was inoculated *via* stomates on the abaxial surface, bacteria were never found in intercellular spaces but exclusively within xylem vessels. The bacteria within invaded host cells showed considerable variation in morphology.

Light micrographs of infected stem sections obtained 20 days after inoculation showed lytic cavities in the vascular tissue (Plates 41A, 41B).

2.2. Intercellular invasion

In stems infected with *Xcm*, bacterial cells were present in intercellular spaces but only in cells associated with vascular tissue (Plates 36A, 36C, 37A). Although large intercellular spaces were available in other types of cells (eg. chlorenchyma) (Plate 40B) no bacterial cells were observed in them. Also no bacteria were detected in the intercellular spaces of infected leaf tissue. Thus movement of *Xcm* appears to be mainly in xylem vessels with much lower numbers in intercellular spaces.

2.3.Fibrillar material

Large amounts of dense, fine-structured fibrillar material which is likely to be extracellular polysaccharide(s) (EPS) (see Discussion IV) secreted by the bacteria was invariably associated with bacterial cells present in both xylem tissues and in intercellular spaces (Plates 37A-37H). This material varied considerably in appearance from almost amorphous to more organized fibrils, occluded the entire lumen of infected cells and was often associated with disruption of pit membranes as bacteria and EPS flowed between host vessels. Sometimes, the bacterial cells were surrounded by an electron-lucent halo.

2.4.Deposits

A relatively thick, electron dense, granular material(s) was deposited onto cell walls and intercellular spaces adjacent to infected cells in both stems and leaves (Plates 38A-38I). Electron dense material also appeared on the inside of vessels and of intercellular spaces between xylem vessels-xylem parenchyma and between xylem parenchyma cell walls. In vessels the material often occlude pits. The multilayered appearance suggested deposition was in several stages (Plates 38A, 38C, 38H, 38I). The intercellular spaces, cell walls and vessels of uninoculated stem were free of any deposits or fibrillar material (Plates 34A-34F). Some

deposits formed an opaque coating inside of xylem parenchyma cell walls which could be a protective layer (Robb et al., 1979) (Plates 35K,38B).

2.5.Host responses

Xylem vessels in infected tissues became plugged with material other than bacterial cells and EPS. These materials were often seen in vessels that were evidently not colonized by the pathogen. Plugging material such as gels were apparently produced in xylem parenchyma cells and secreted into the adjacent xylem vessels (Plate 39A). Gels and tyloses were observed in the free hand and semithin sections (Plates 39B, 41A, 41B).

2.6.Other cells

a).Stems - Cells distal from heavily infected vascular tissue seemed unaffected and their walls and organelles were intact. Although there were large intercellular spaces available, they were free of bacterial cells or deposits (Plate 40B). These cells were similar in appearance to their counterparts in uninfected tissue (Plates 34A).

b).Leaves - Most of the mesophyll and pallisade cells surrounding infected vascular tissue were deformed; the organelles had degenerated and the cell walls had collapsed but no cell wall degradation was evident

(Plates 40D-40G). However, there were a few cells adjacent to infected xylem vessels that seemed to be unaffected and intact (Plates 35D, 35J).

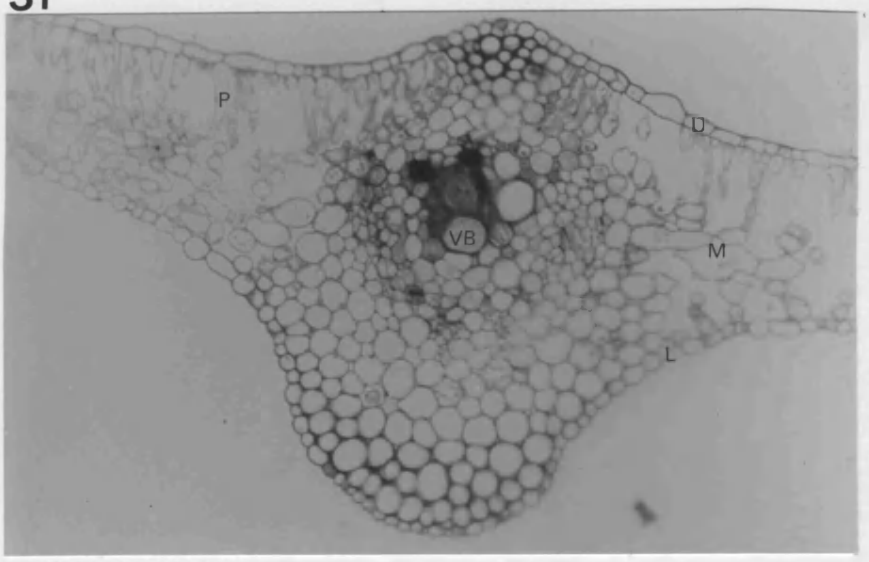
1.Uninoculated tissue -Plates 31-34F

Plate 31- Light micrograph of cassava leaf (TS) showing the general distribution of cells. Upper (U) and (L) epidermis, Pallisade cells (P), Mesophyll cells (M), Vascular bundle (VB).

Plate 32 A-C- Electron micrographs of uninfected cassava leaf tissue.

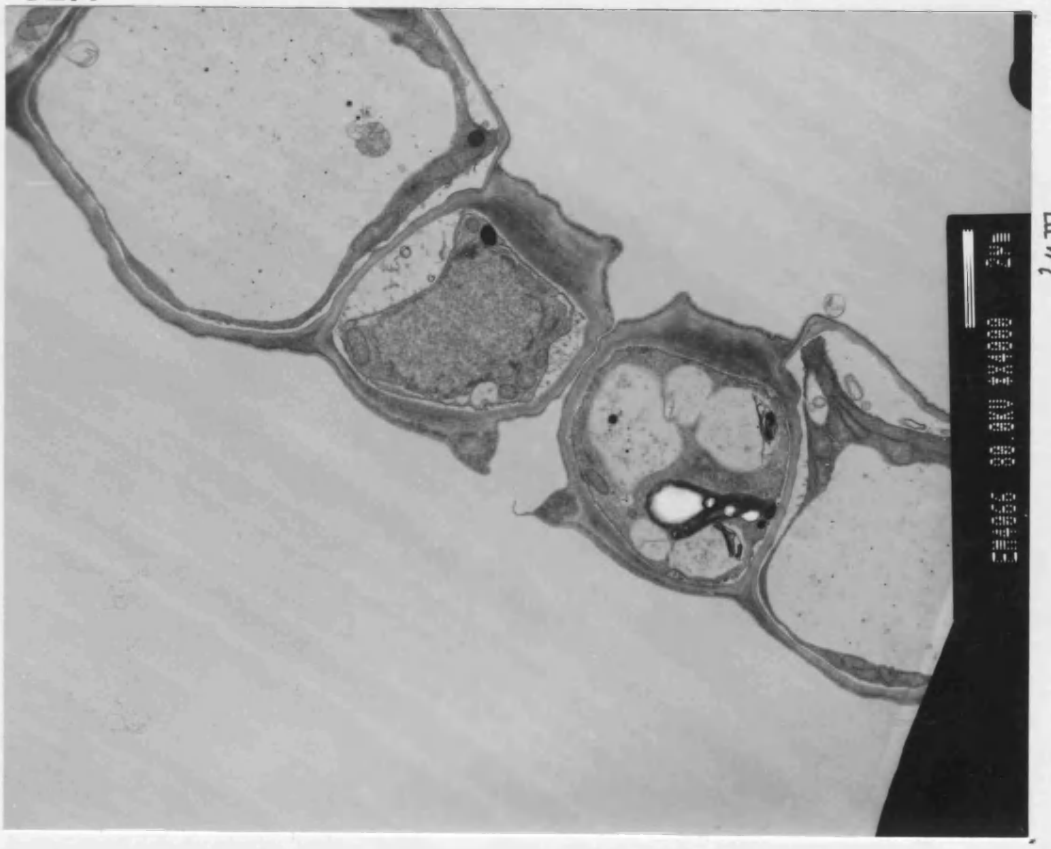
Plate 32A- Upper epidermis with a stoma (the stomatal pore is a likely point of entry for the pathogen in natural infections.

31



30μm

32A

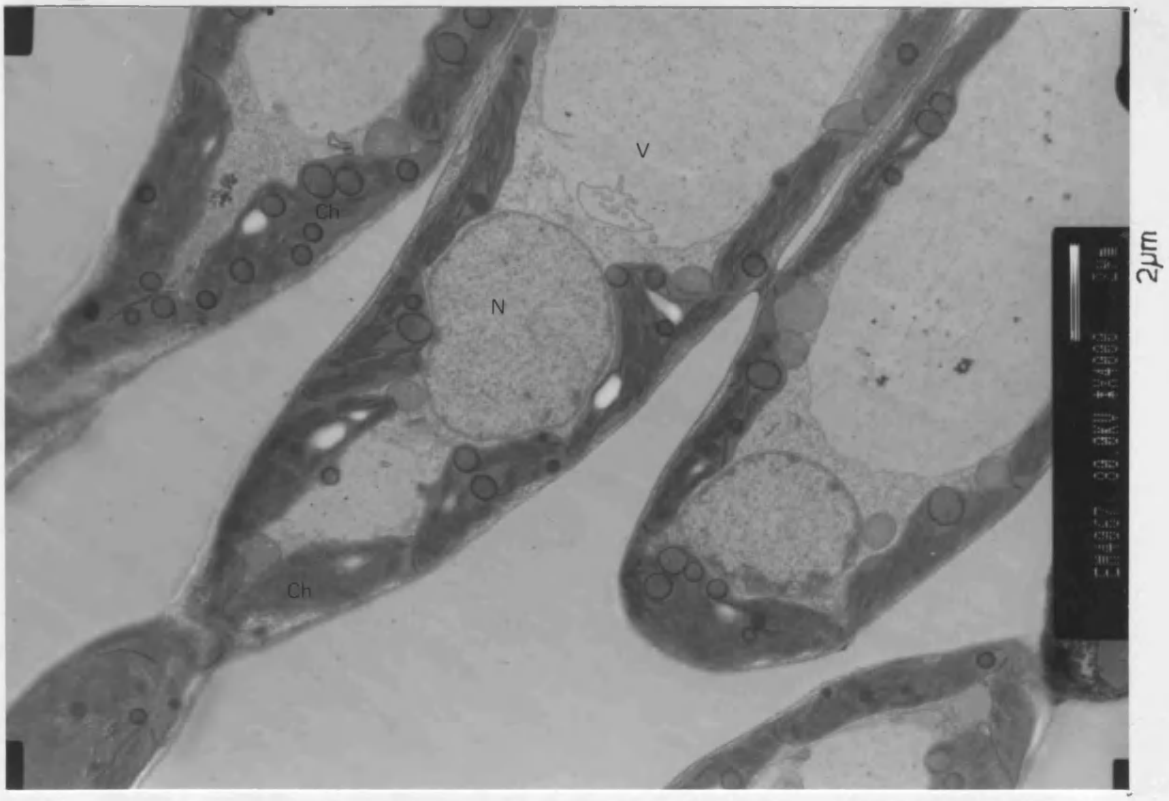


2μm

Plate 32B- Pallisade parenchyma cells showing large nucleus (N), abundant chloroplasts (Ch) and a vacuole (V).

Plate 32C- Mesophyll cells. Note large intercellular space (IS).

32B



32C

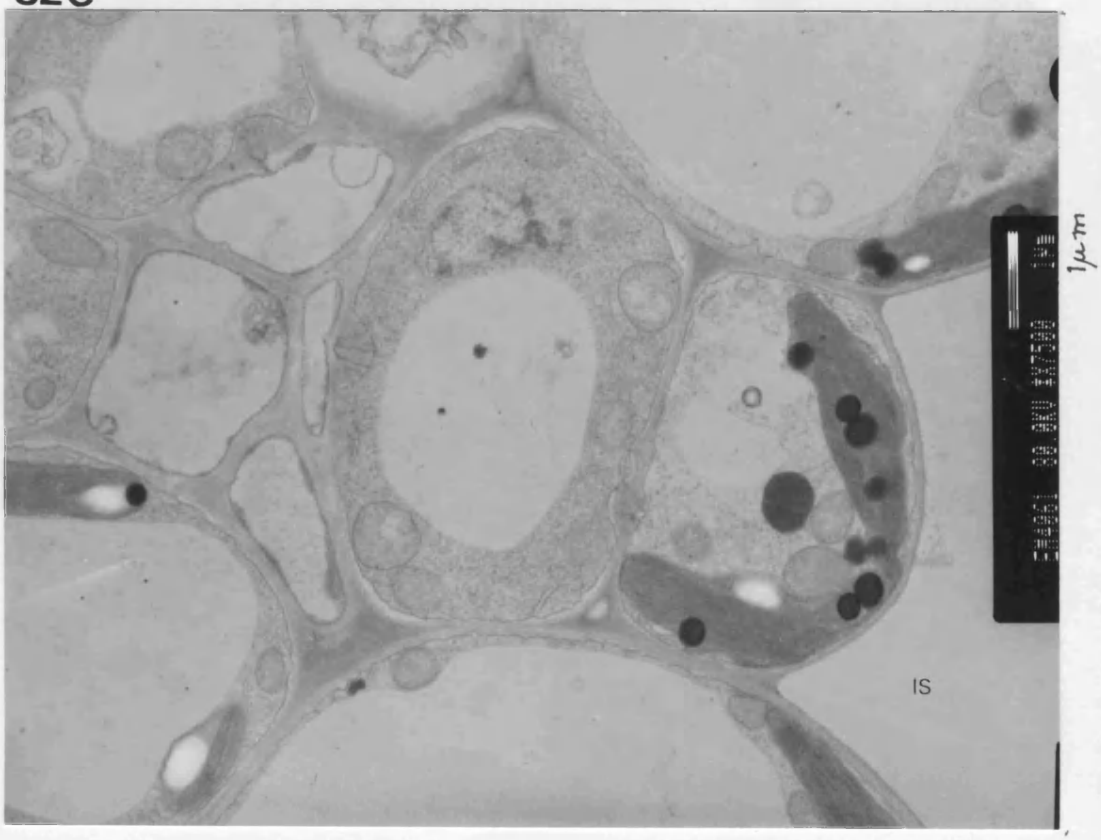
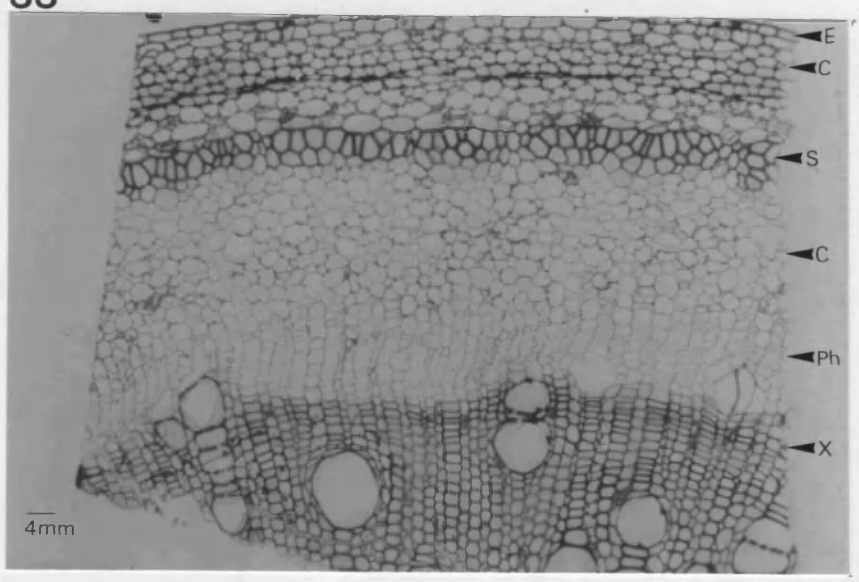


Plate 33- Light micrograph of cassava stem (TS) showing the general distribution of cells. Epidermis (E), Chlorenchyma (C), Sclerencyma (S), Chlorenchma (C), Phloem (Ph) and Xylem (X).

Plates 34A-34F- Electron micrographs of uninfected cassava stem tissue.

Plate 34A- Healthy chlorenchyma cells. Note the large intercellular spaces (IS) between cells.

33



34A

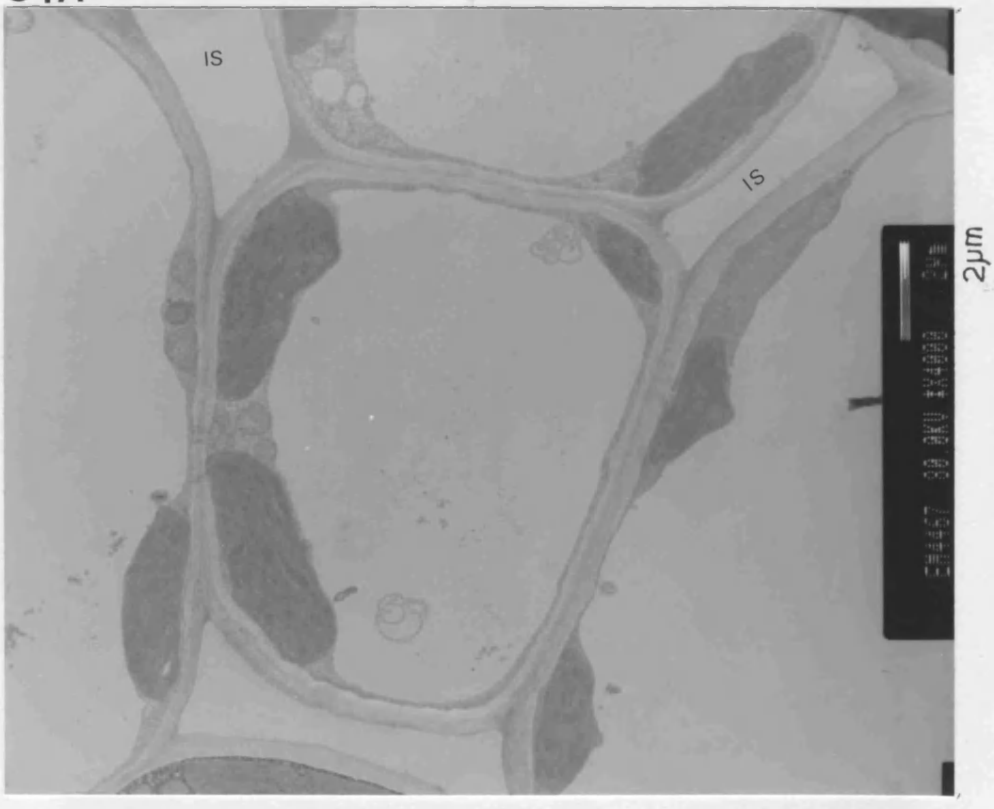
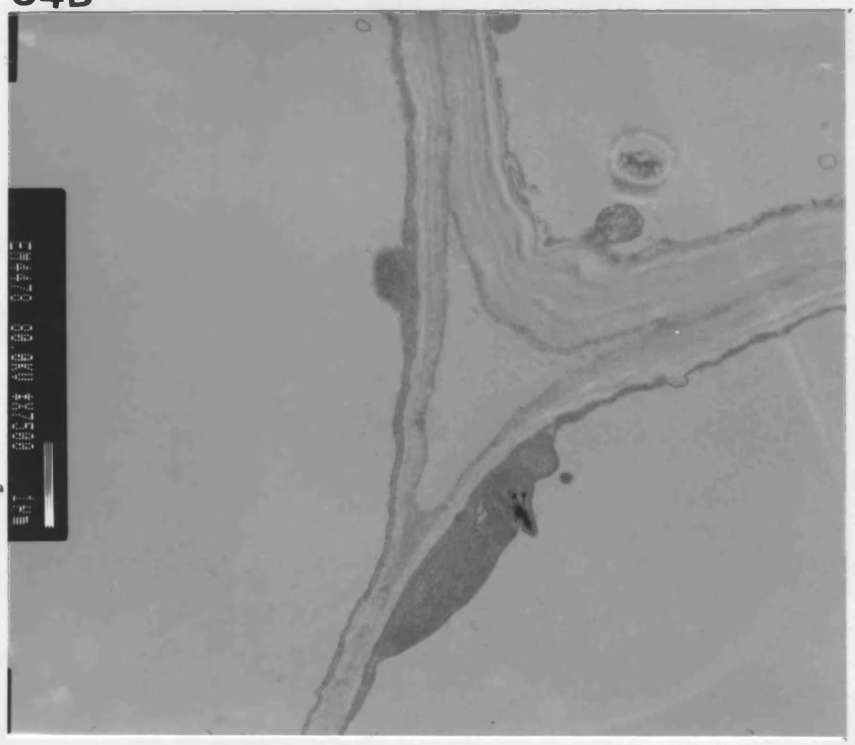


Plate 34B- Intercellular space between xylem parenchyma cells.

Plate 34C- General view of xylem with pits between vessels (➤) and between vessels and xylem parenchyma cells (⟶).

34B



34C

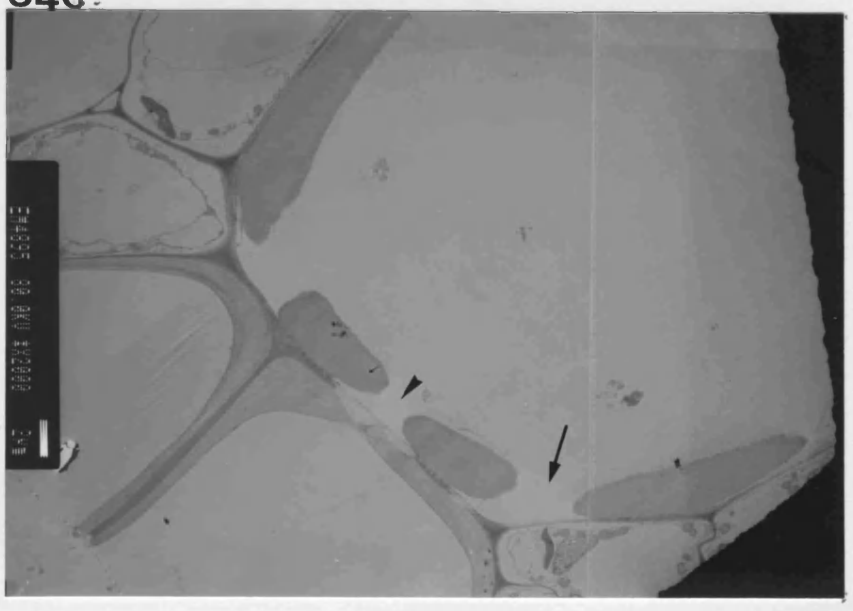
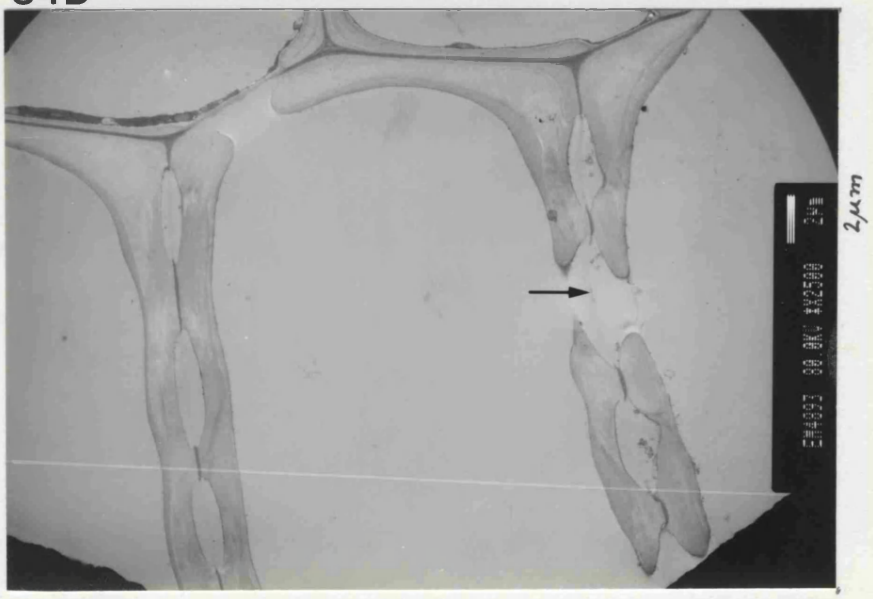


Plate 34D- Pits connecting three xylem vessels. Note that only faint traces of pit membrane remain (⟶).

Plate 34E- Pit between a xylem vessel (XV) and a Xylem parenchyma cell (XP). Note the protective layer (➤) secreted by the xylem parenchyma cell.

34D



34E

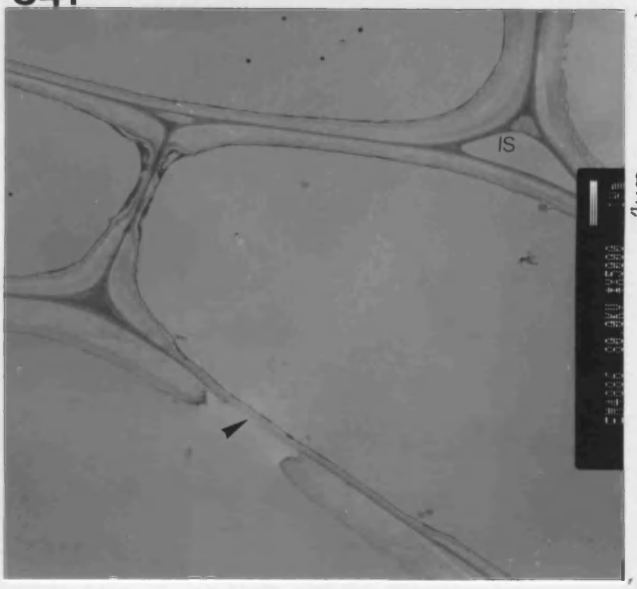
Plate 34F- General view of xylem tissue. Note the pit (►) between the vessel and xylem parenchyma cell (XP) and the clear intercellular space (IS).

2.<u>Vascular invasion (Stem)</u>-Plates 35A-35E

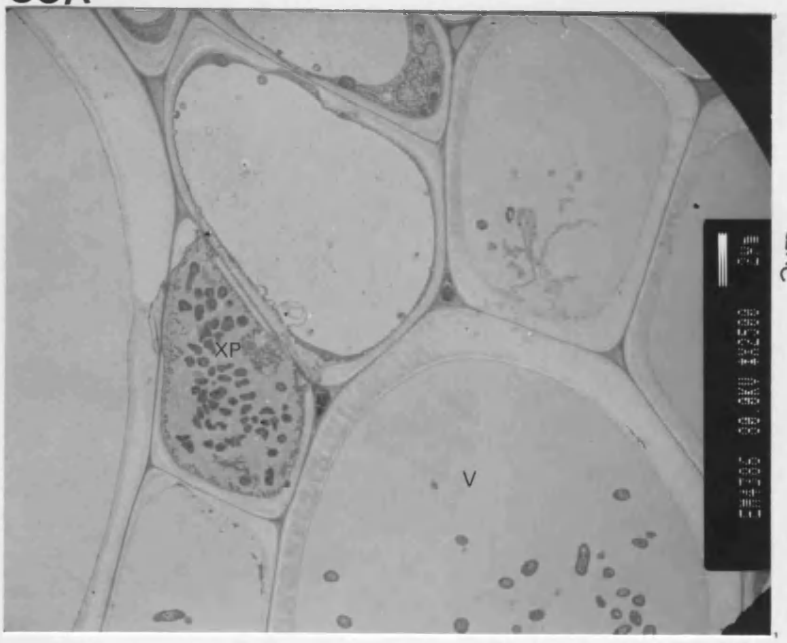
Plate 35A- General view of infected xylem tissue. Note bacteria in vessels (V), xylem parenchyma (XP) cells and intercellular spaces. Cytoplasm of invaded xylem parenchyma cell is disrupted.

Plate 35B- Detailed view of xylem parenchyma cell with bacteria embedded in fibrillar material. Note electron dense material adjacent to the inside wall; thickened protective layer (►) of adjacent parenchyma cell and withdrawal and vesiculation of cytoplasm (cy).

34F



35A



35B

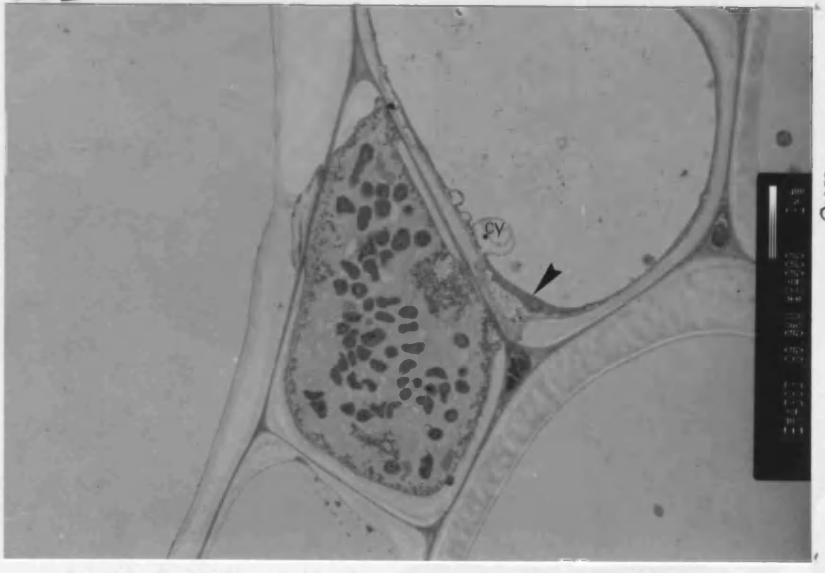
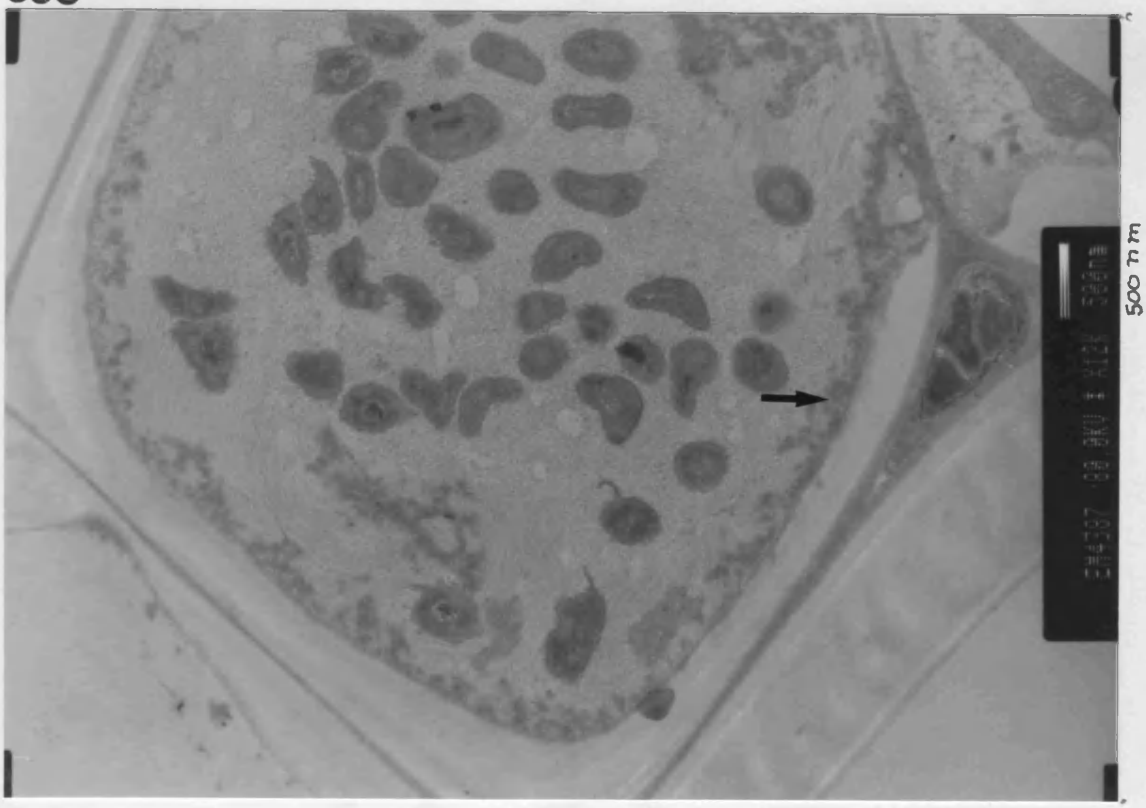


Plate 35 C- Bacteria embedded in fibrillar material in a xylem parenchyma cell. Note grannular material deposited on the inside wall (➡). Bacterial cell in the intercellular space embedded in fibrillar material.

Plate 35 D- Bacterial cells in an infected vessel. Note that one pit membrane is absent (⟶); adjacent xylem parenchyma cell (XP) appears unaffected; bacteria are highly pleiomorphic.

Plate 35 E- Bacteria in fibrillar material appearing to flow through a pit between two vessels

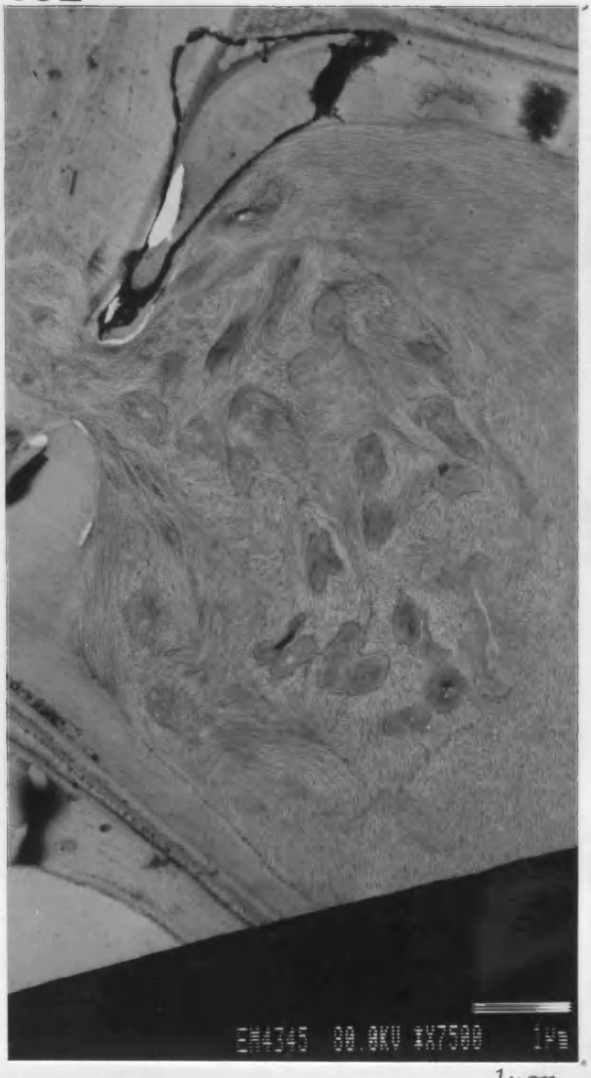
35C


500 nm

35D


XP
EM4390 80.0KV X4000 2µm
2µm

35E


EM4345 80.0KV X7500 1µm
1µm

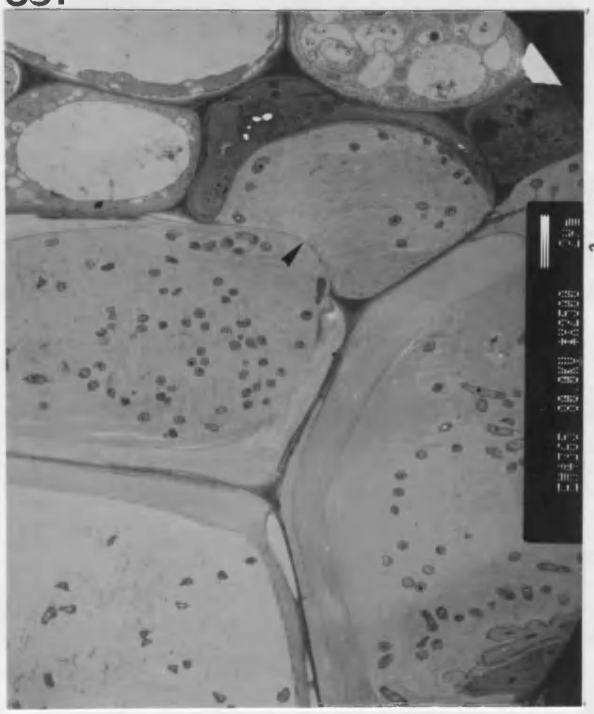
3.Vascular invasion (Leaf)-Plates 35F-35N

Plate 35F-35K- General and detailed views of xylem containing bacteria embedded in fibrillar material (presumably bacterial EPS).

Plates 35F & 35G- Growth pressure is apparent as the contents of one vessel distort a vessel-vessel pit membrane (►).

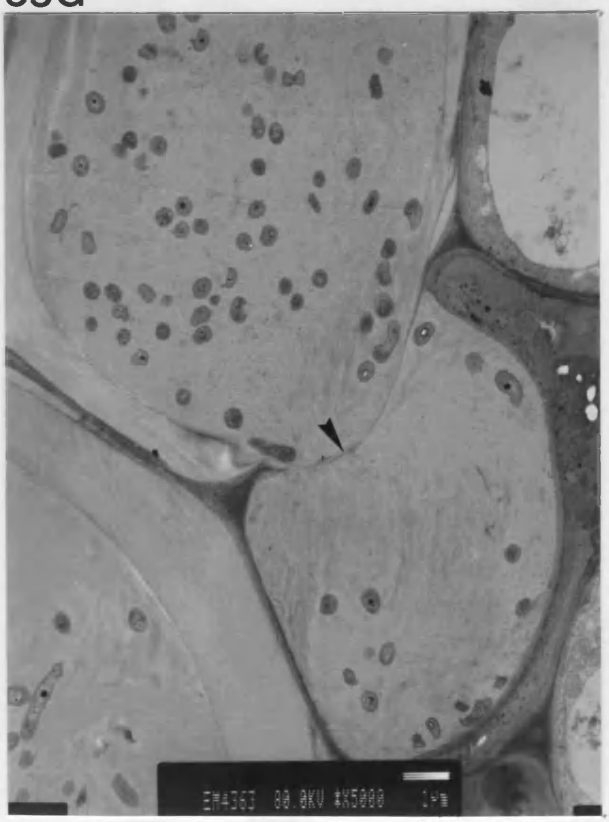
Plate 35H- Infected xylem vessels. The pit membrane appears to be occluded with a granular deposit (⟶). the adjacent xylem parenchyma cell (XP) is in an advanced state of necrosis.

35F



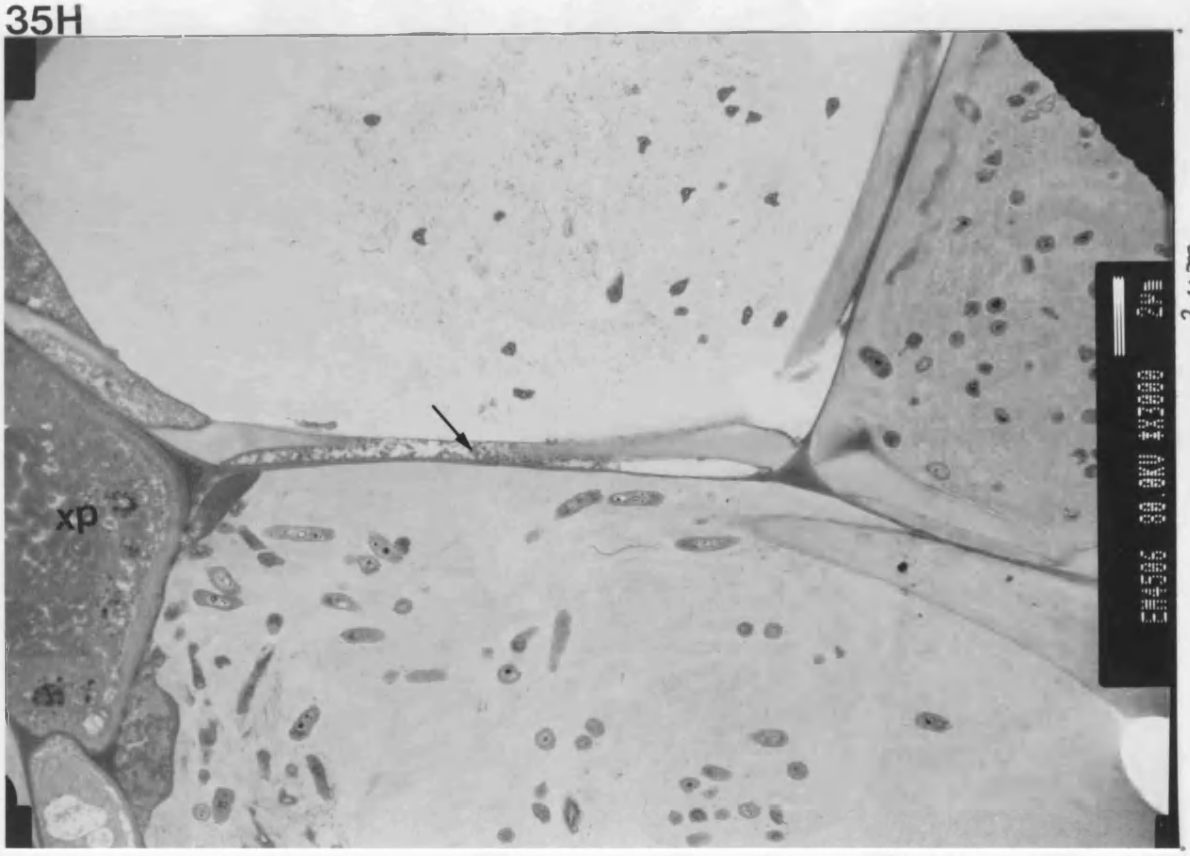
2μm

35G



1μm

35H

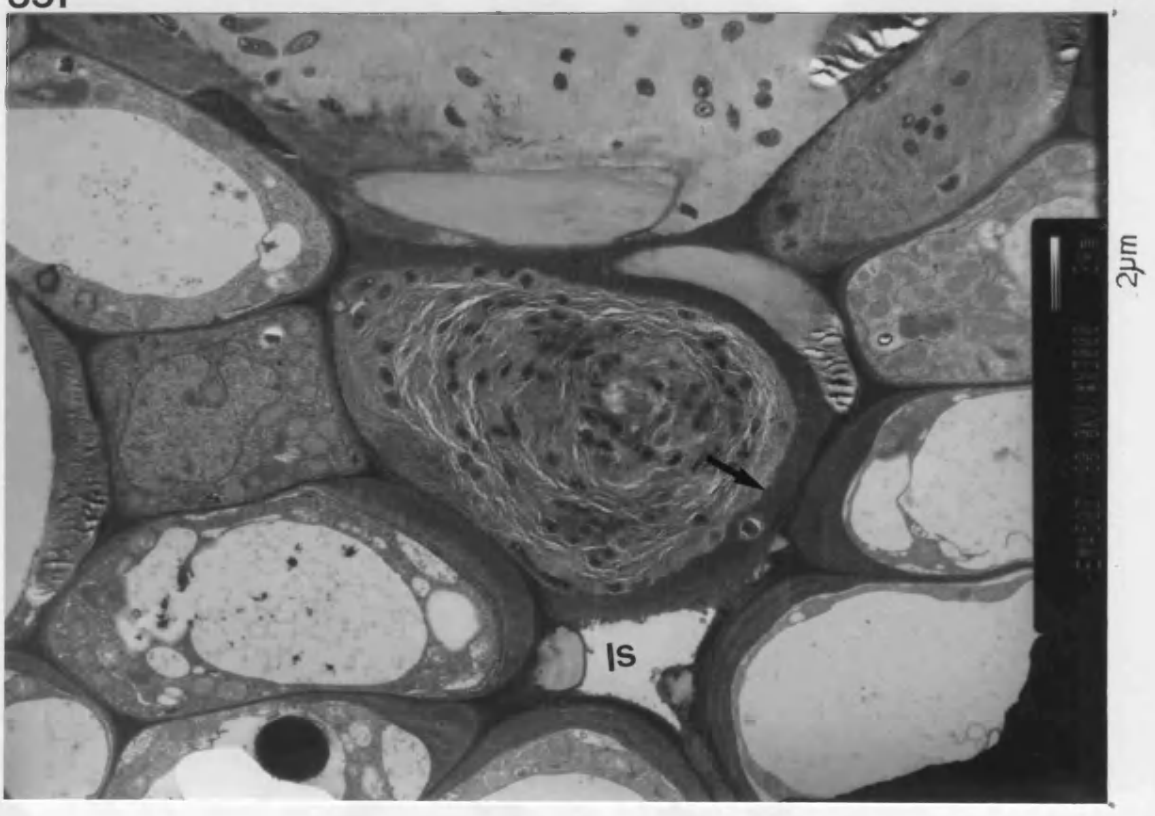


2μm

Plate 35I- Note the thick layer of electron opaque material deposited on the inside wall of cells containing bacteria (⟶) and also in intercellular space (IS). All other cells without bacteria are degenerating and highly vacuolated.

Plate 35J- Note the difference in texture of the fibrillar material in the two infected xylem vessels, electron dense dark deposits on cell walls and the necrotic cell. The parenchyma cell (XP) appears unaffected.

35I



35J

Plate 35K- Bacterial cells in vessels surrounded by two types of fibrillar material (f1 & f2). Electron opaque material is deposited (⟵) on the inside of an infected xylem vessel cell with bacteria and a similar material is in the intercellular space.

Plate 35L- All cells with and without bacteria are in an advanced stage of degeneration. p1 and p2 pit membranes either dissolved or physically disrupted enabling movement of bacteria between cells. Dark granular material has been deposited on the vessel wall (◄).

35K

35L

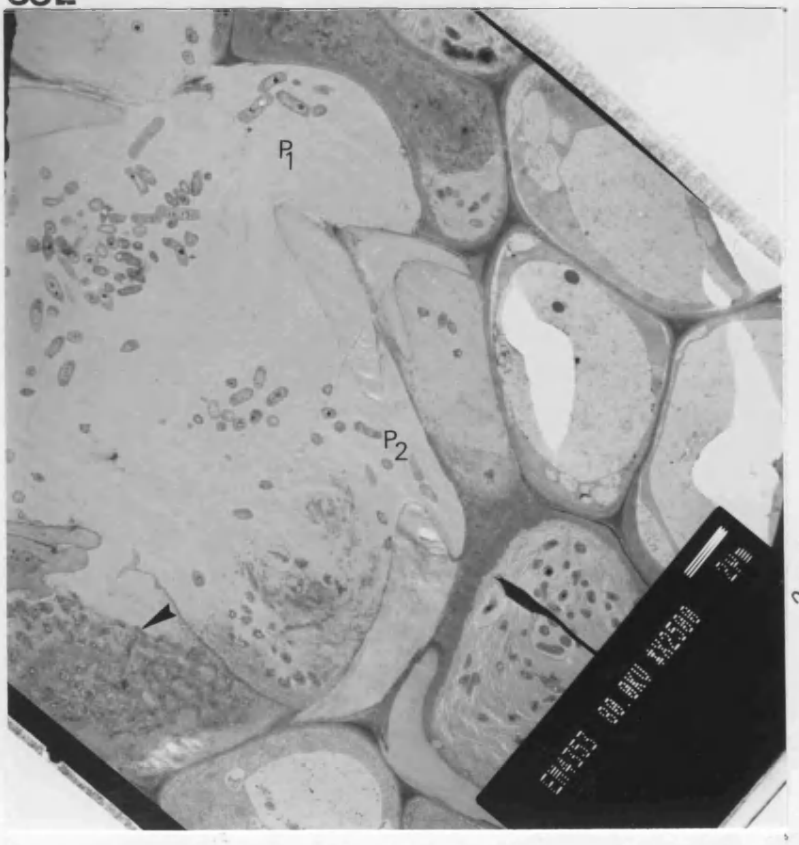


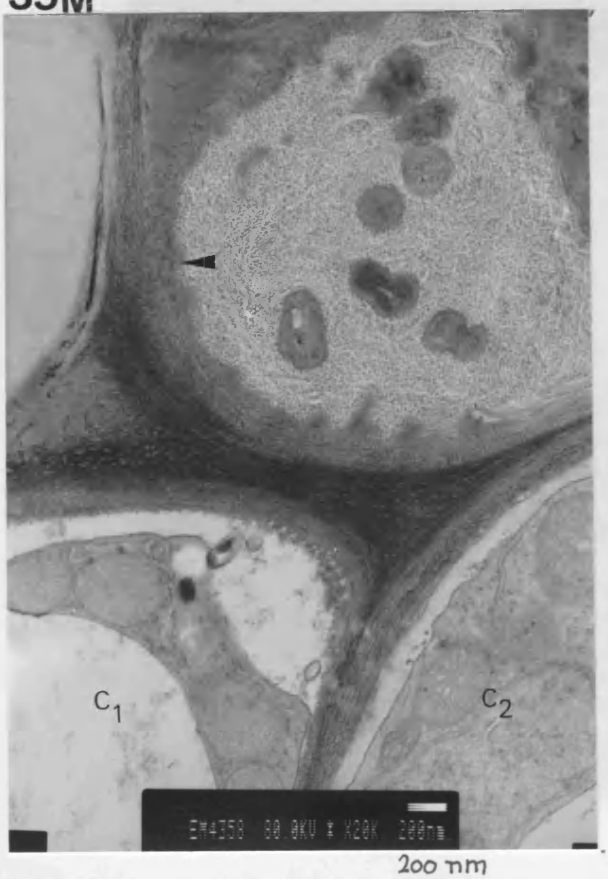
Plate 35M- Xylem parenchyma cell with bacteria embedded in fibrillar material. Note the amorphous, convoluted layer deposited on the inside of the wall. Adjacent C1 cell is in an advanced stage of degeneration; in C2 the cytoplasm is separating from the cell wall; in both these cells the walls contain electron dense material.

Plate 35N- LS of xylem tissue with bacteria. In spite of the extensive area of pit membranes, bacteria remain confirmed to one of the two large vessels . Note the movement of bacteria between other disrupted pit membranes.

4.<u>Intercellular invasion of stem</u>-Plates 36A-36C

Bacterial cells in intercellular space embedded in fibrillar material. Note that adjacent cells degenerating or responding by vesiculation. Large globules (lipid?) are present in cytoplasm of two cells .

35M

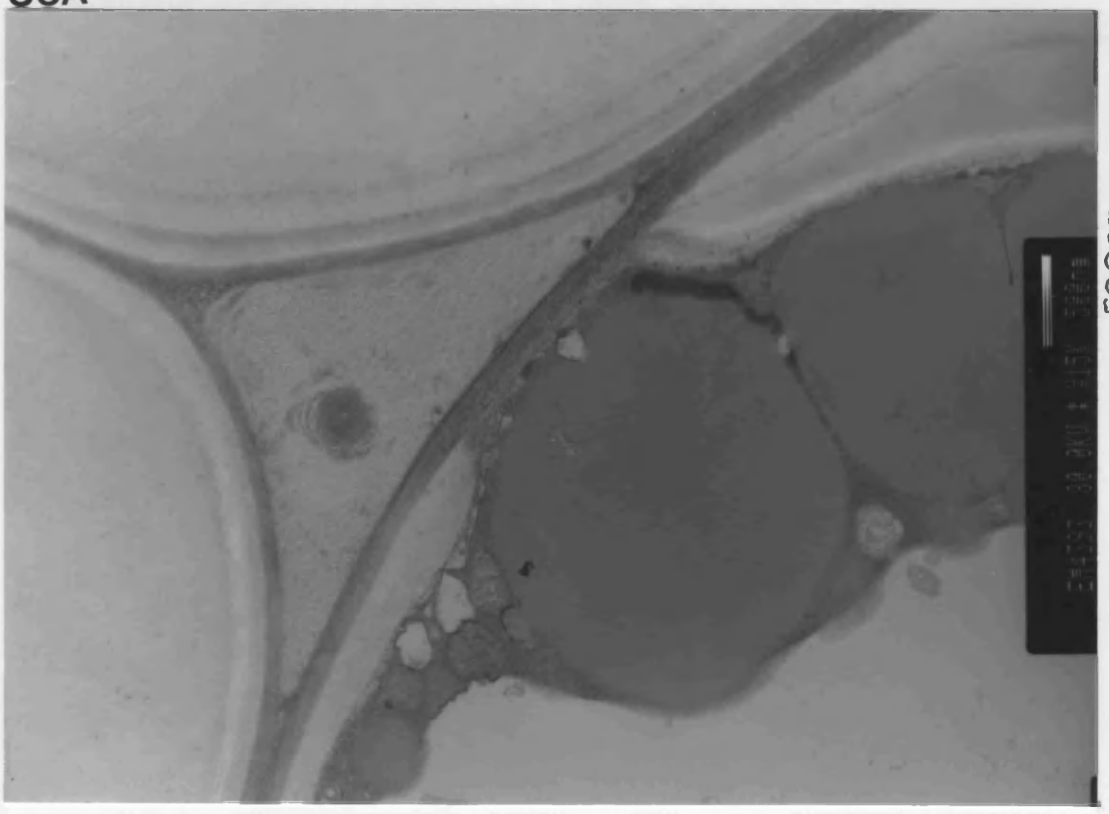


200 nm

35N

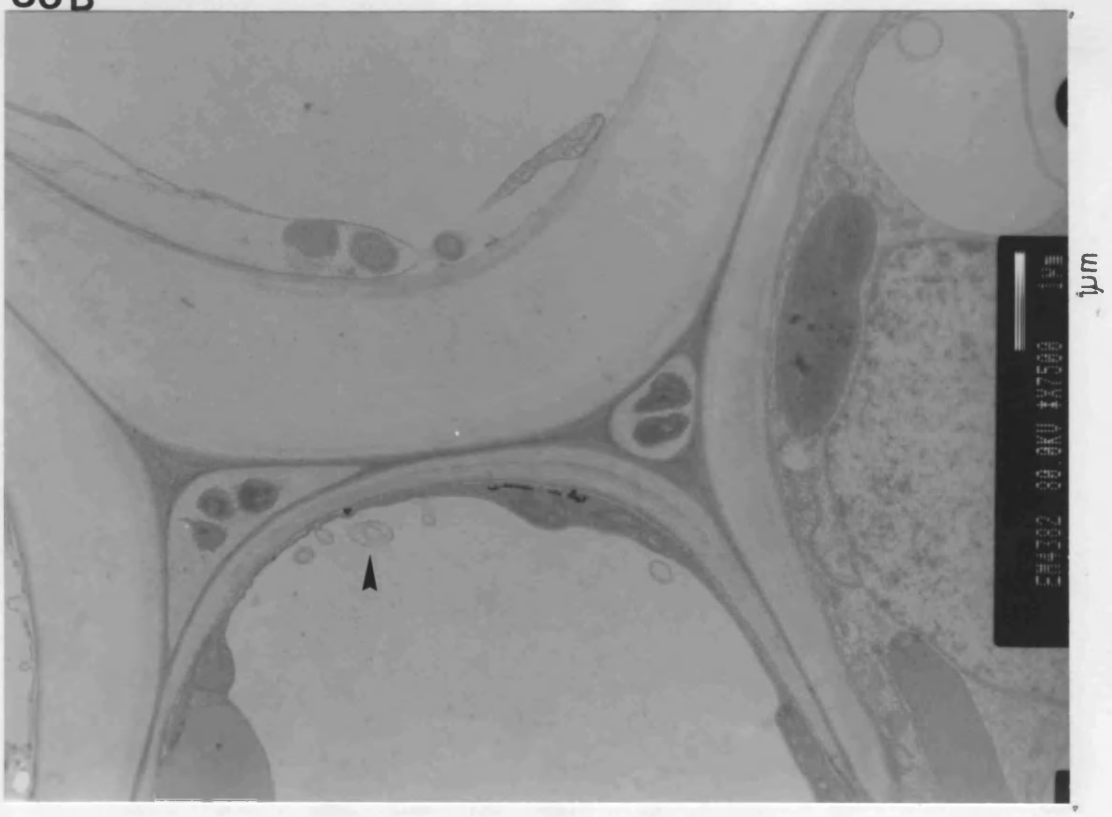
2µm

36A

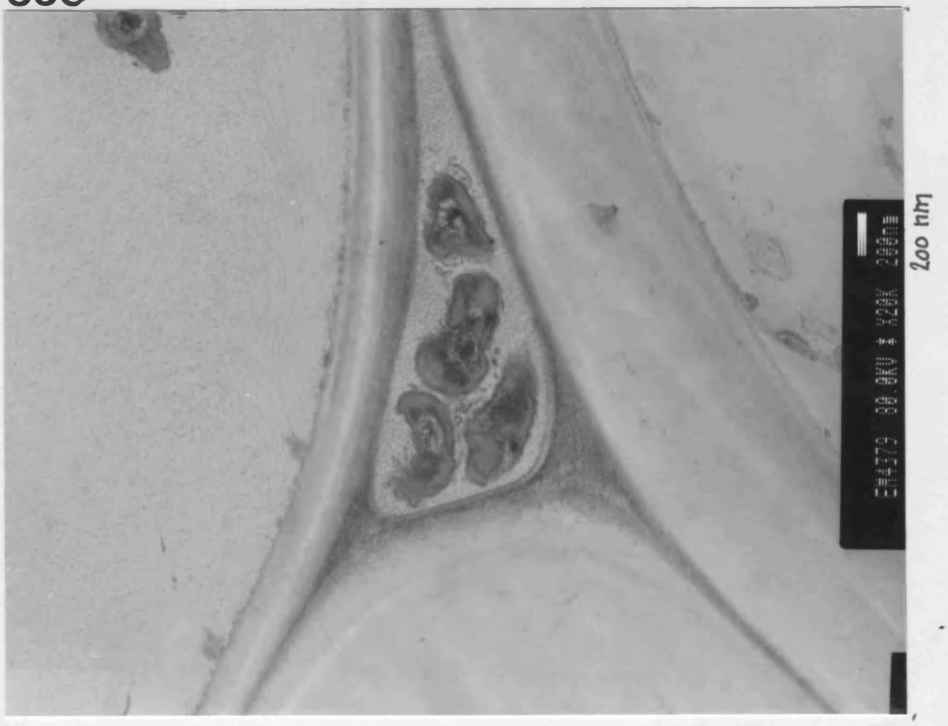


500nm

36B



36C



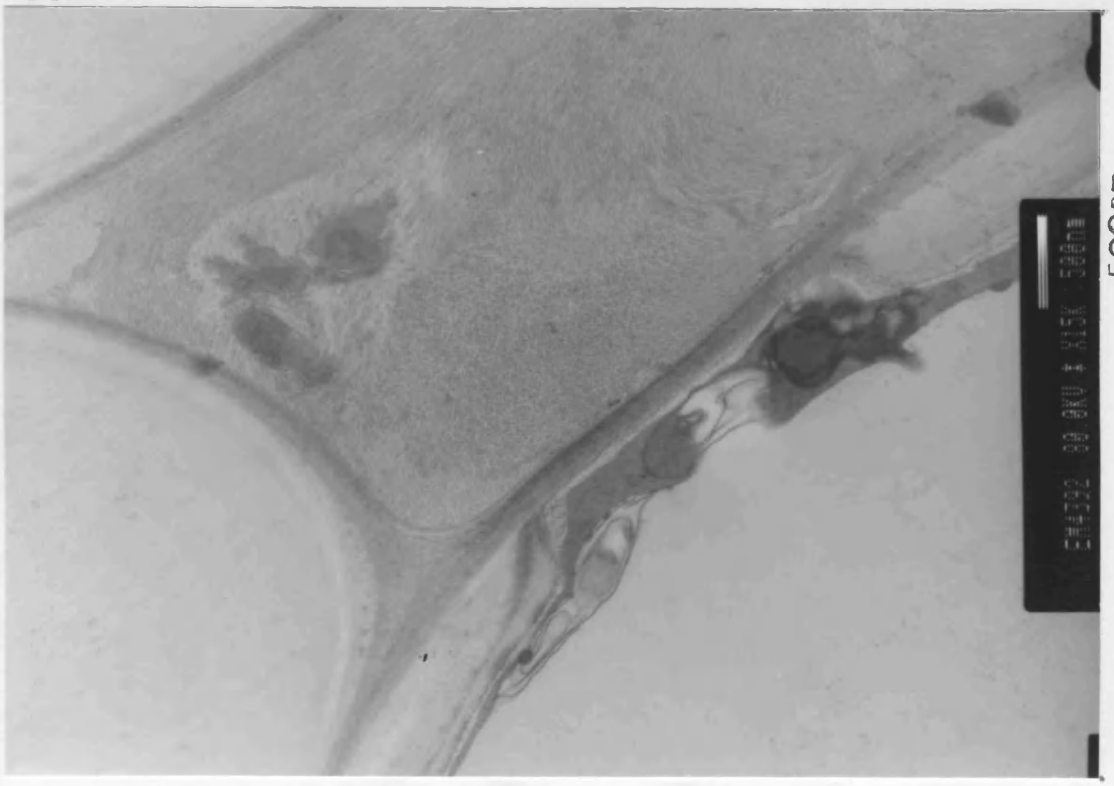
5. Fibrillar material (bacterial EPS?) -Plates 37A-37H

Plates 37A-37B- Stem

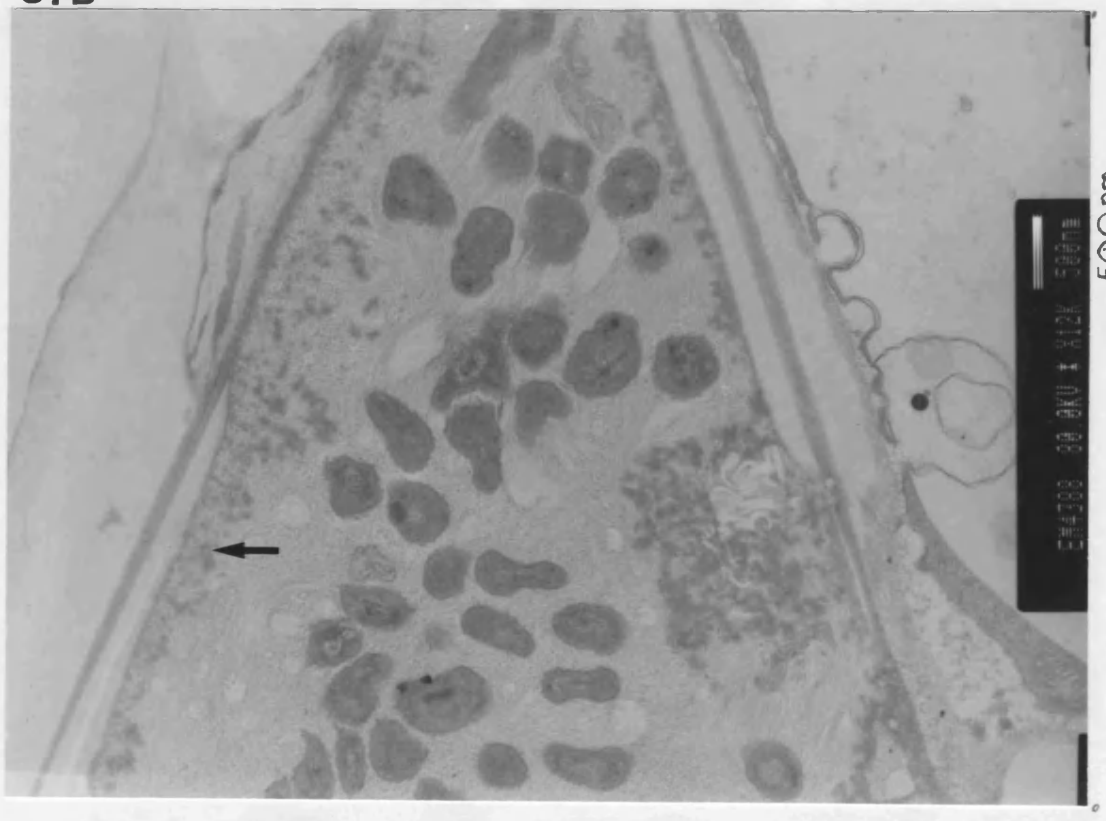
Plate 37A- Bacterial cells embedded in EPS in an intercellular space. Note the slightly electron lucent area around bacterial cells; adjacent cells with degenerated organelles (◄).

Plate 37B- Bacterial cells with a range of varying shapes embedded in EPS. Granular deposits on the inside of cell wall.

37A



37B

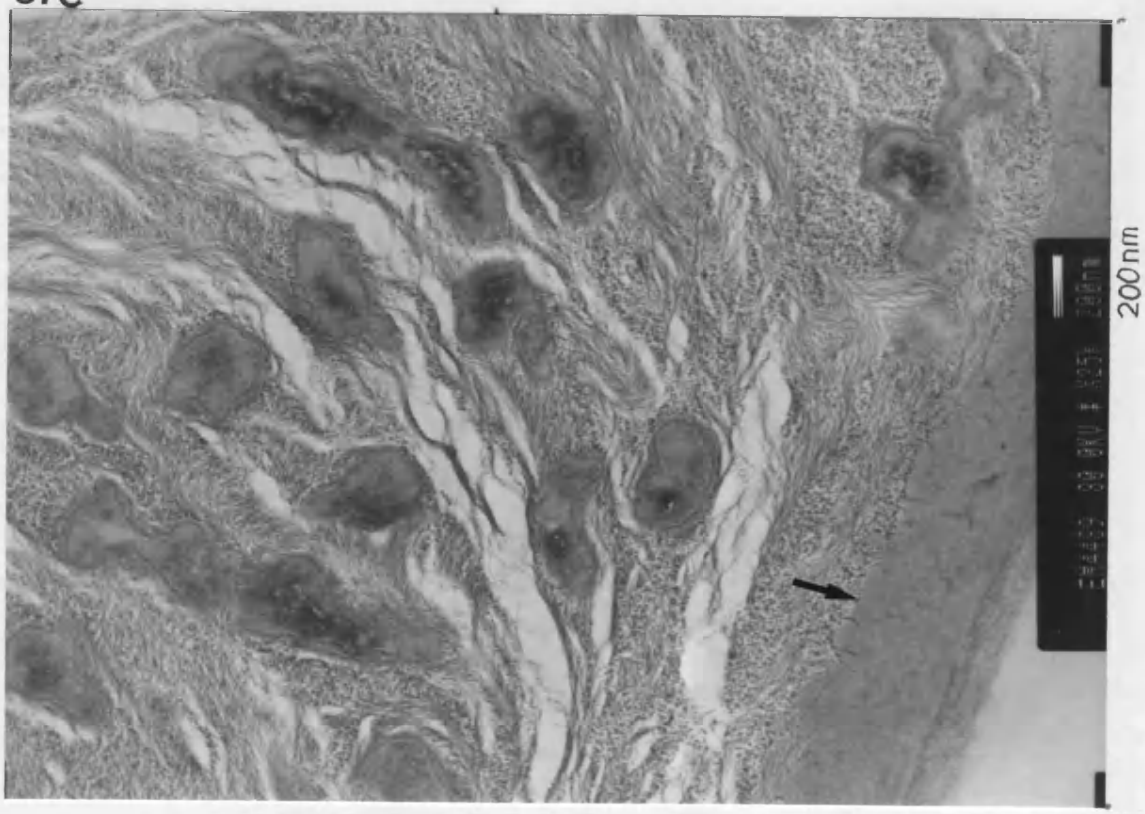


Plates 37C-37H- Leaf

Bacterial cells embedded in EPS.

Plates 37C and 37D- Note the variable morphology of Xcm cells and the electron opaque deposit on the inside of cell wall (⟶).

37C



37D

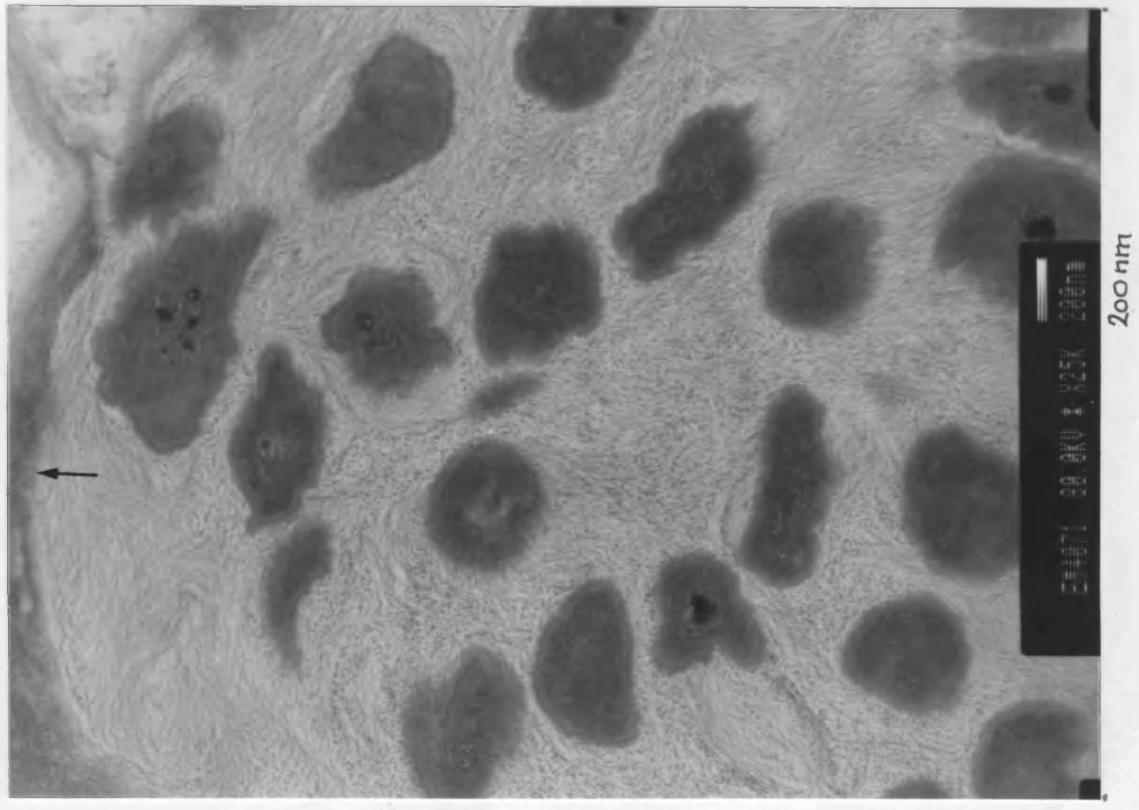
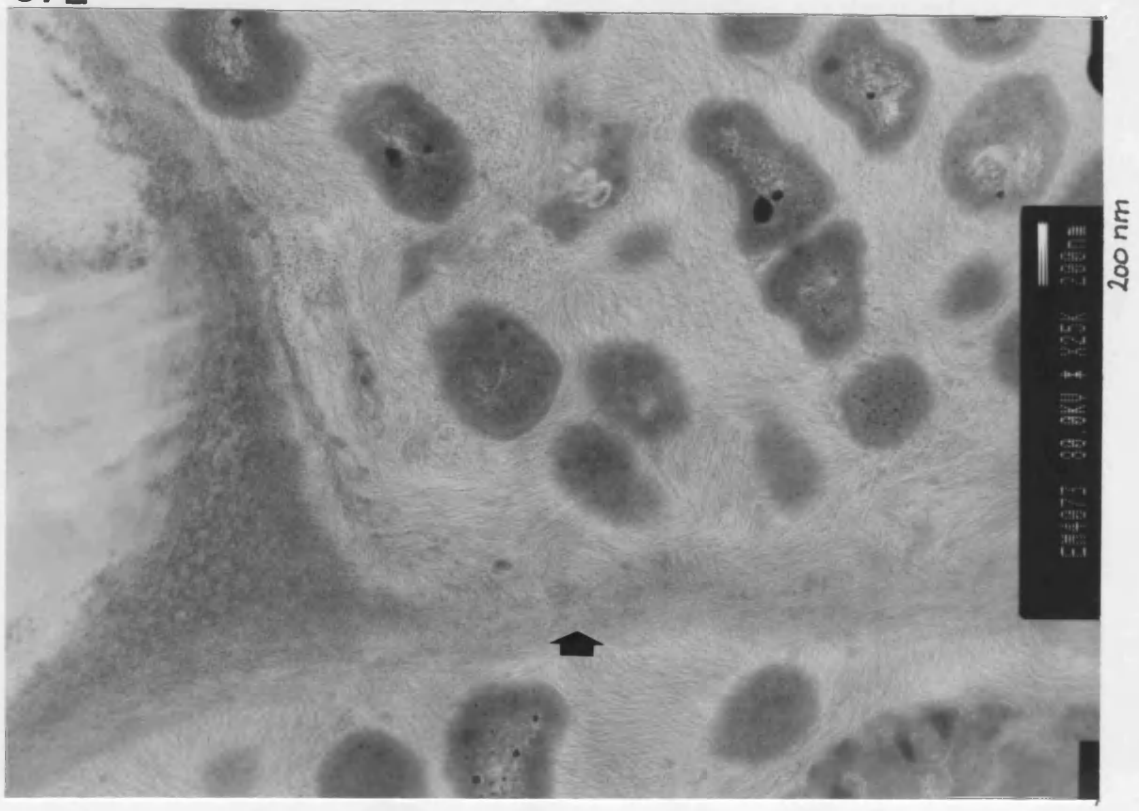


Plate 37E- The cell wall between two adjacent infected cells appears partly disrupted and intimately associated with EPS (➧).

Plate 37F- Note the apparent plasmolysis of adjacent uninfected cell (C).

37E



37F

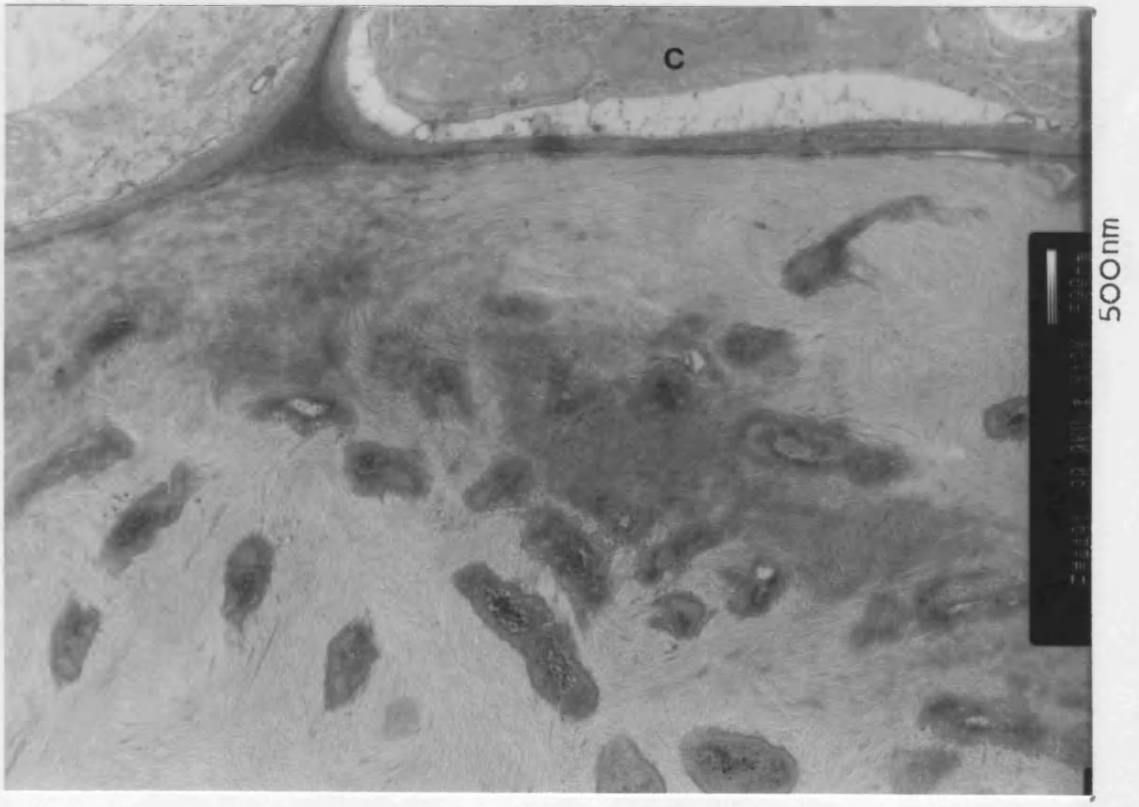
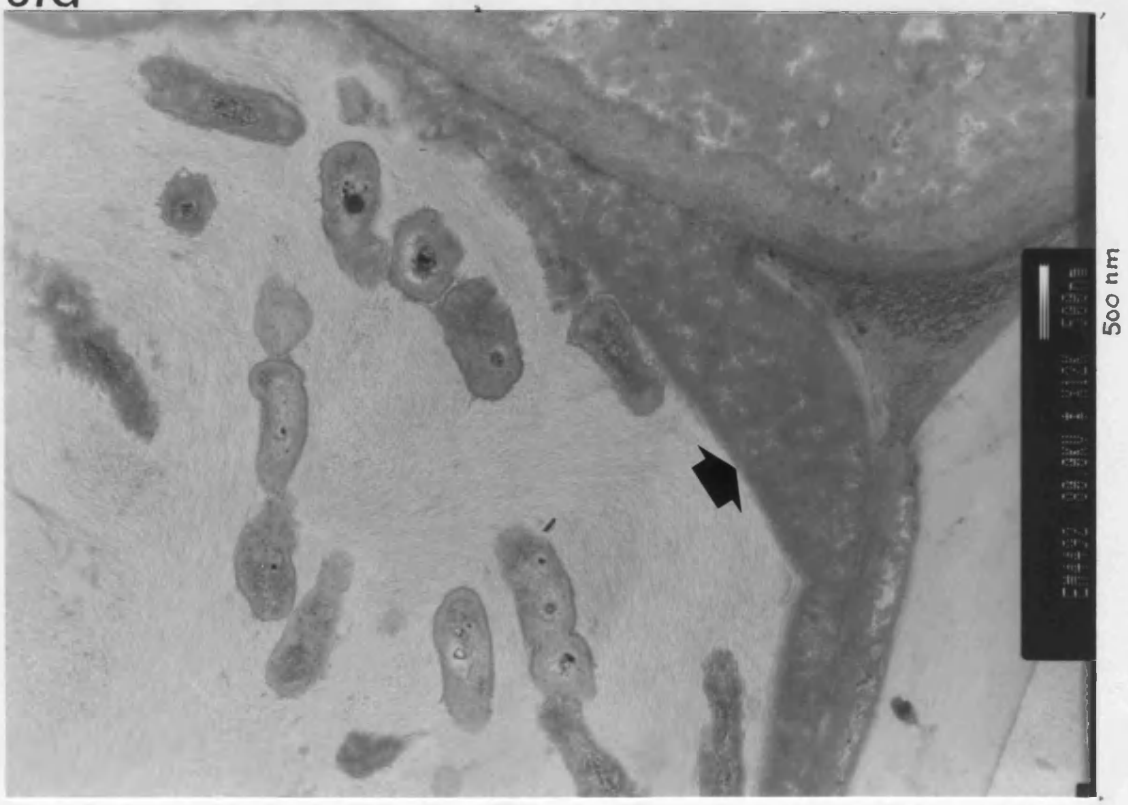


Plate 37G- Dense electron opaque material has been deposited along the inside of the cell wall (➧). The adjacent cell is in advanced state of necrosis.

Plate 37H- Fibrillar material in adjacent infected cells differs considerably (f1 & f2).

37G



37H

6.Deposits -Plates 38A-38I

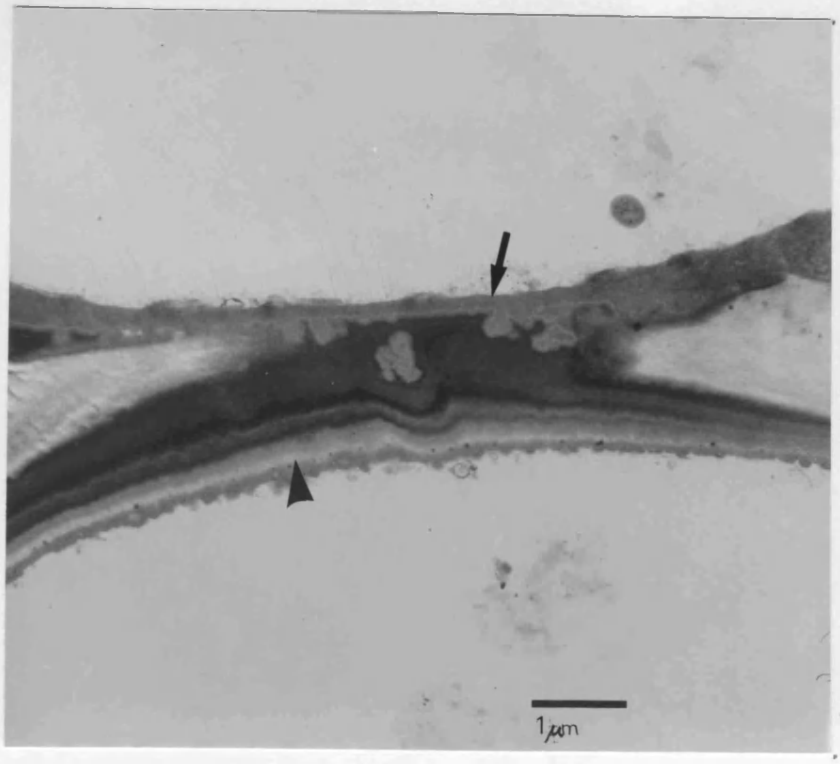
Plate 38A- Stem

Xylem vessel coated in layered electron opaque material. Note smooth (➤) and irregular (➤) vessel lining material. Deposits are thickest at the pit membrane where they occlude the pit.

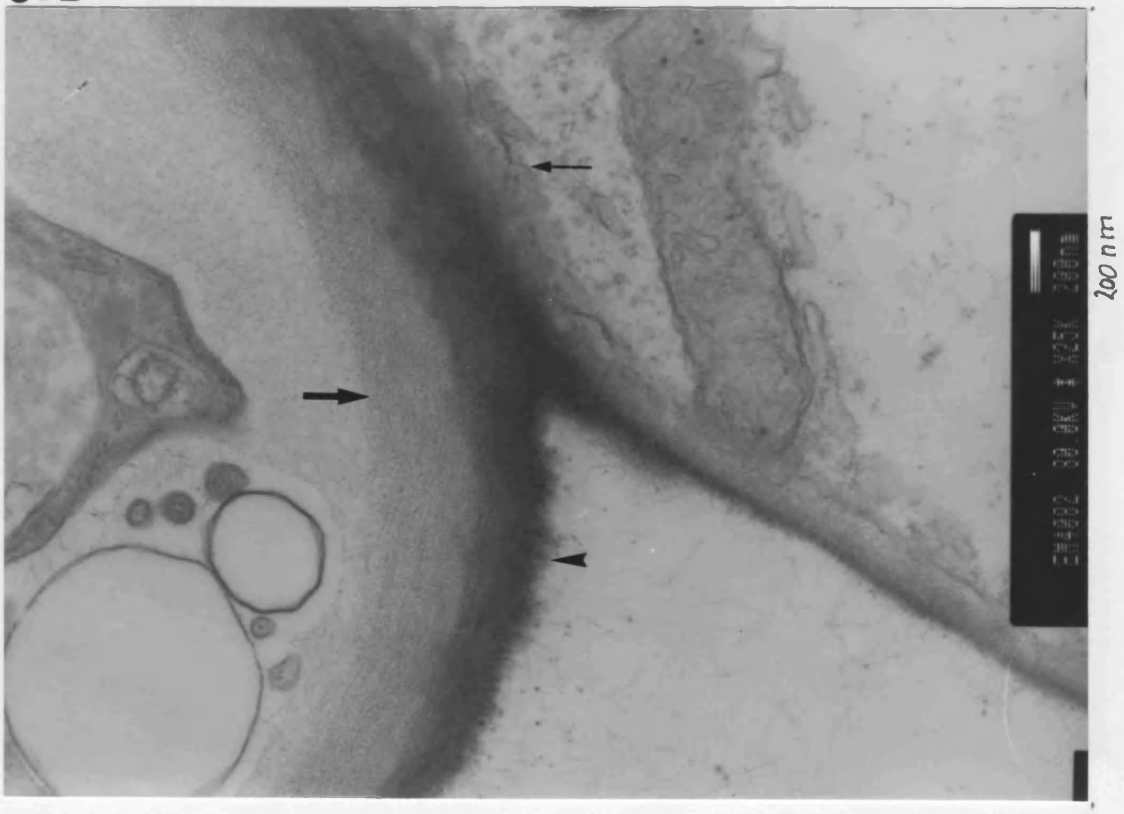
Plates 38B-38I- Leaf

Plate 38B- Electron opaque de posit along outside of cell wall (➤). A protective layer deposited on the inside of the cell wall of two degenerating xylem parenchyma cells (➤).

38A



38B

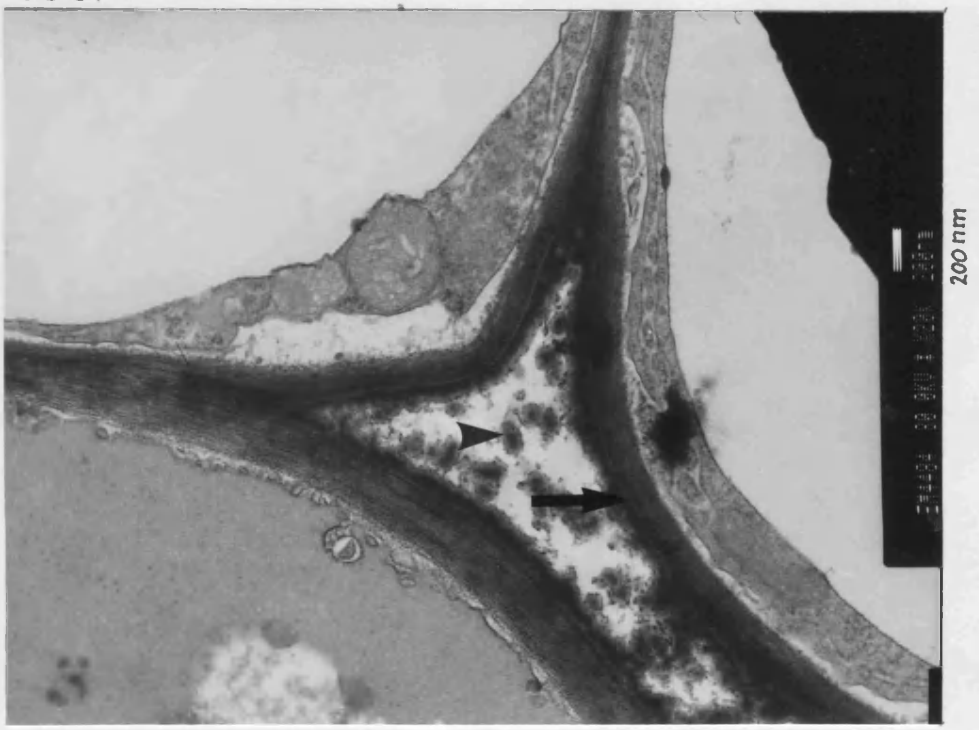


Plates 38C-38E- Electron opaque deposits on cell walls forming intercellular spaces.

Plate 38C- Note the outer granular (➤) and inner smooth (➤) deposits. Adjacent cells show unusual features and their cytoplasm is withdrawn from the cell wall.

Plate 38D- Globular (irregular) deposits.

38C



38D

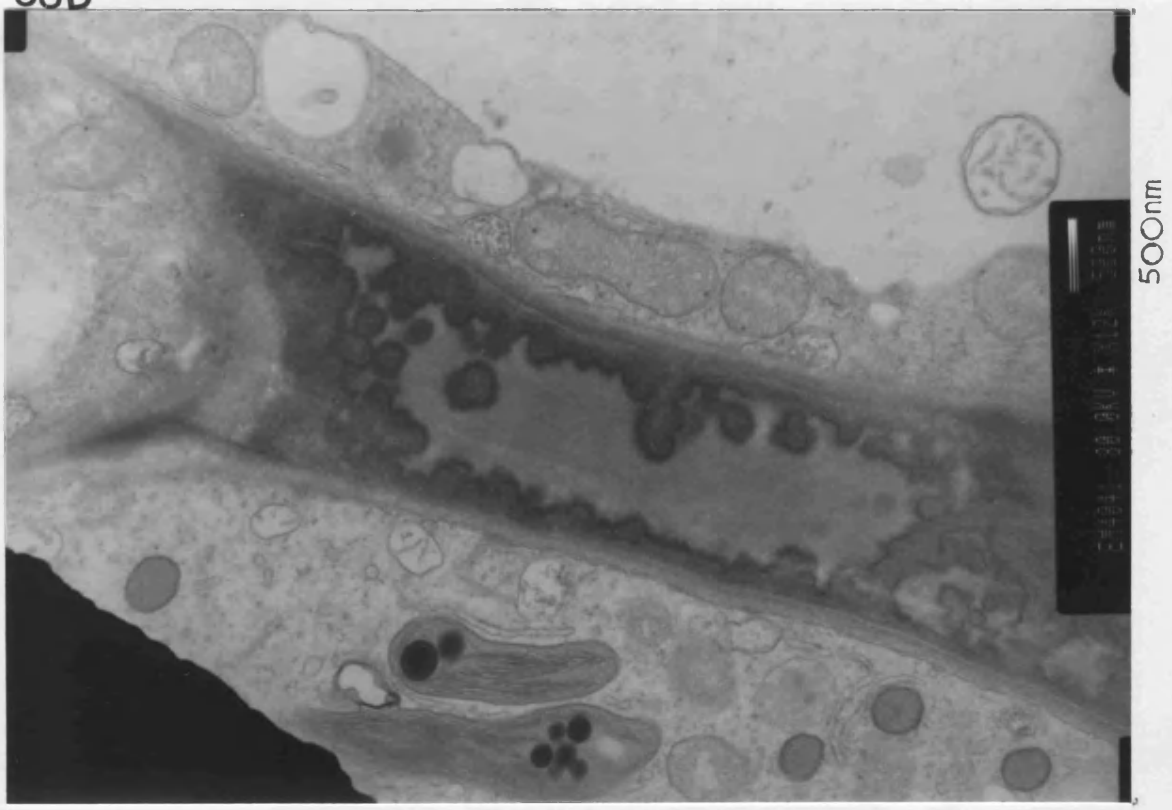
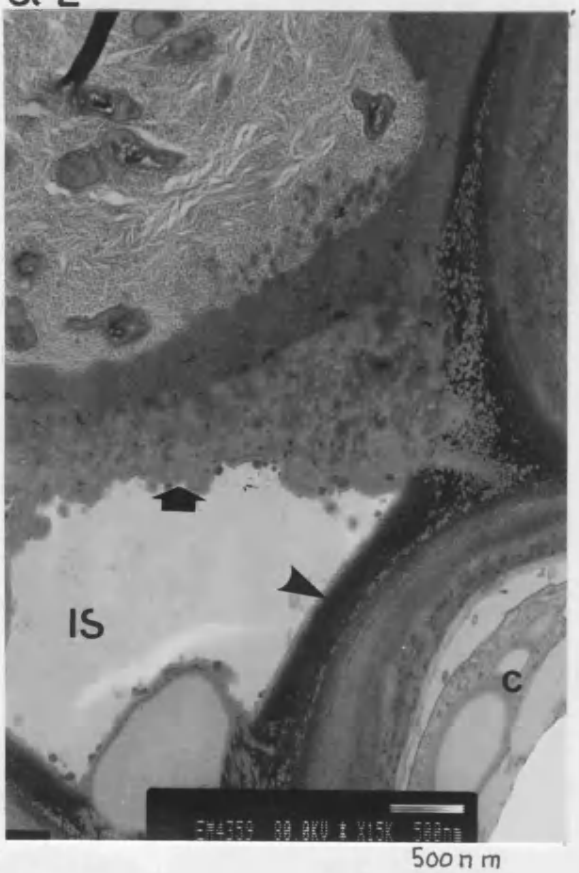


Plate 38E- Smooth (➤) and irregular (➧) deposits within an IS. The cell (C) without bacteria is degenerating; the cytoplasm separating from the cell wall.

Plate 38F- Low power electron micrograph of infected vascular tissue of a cassava leaf. Vessel coating material lines the lumina of four xylem vessels (V1, V2, V3 and V4).

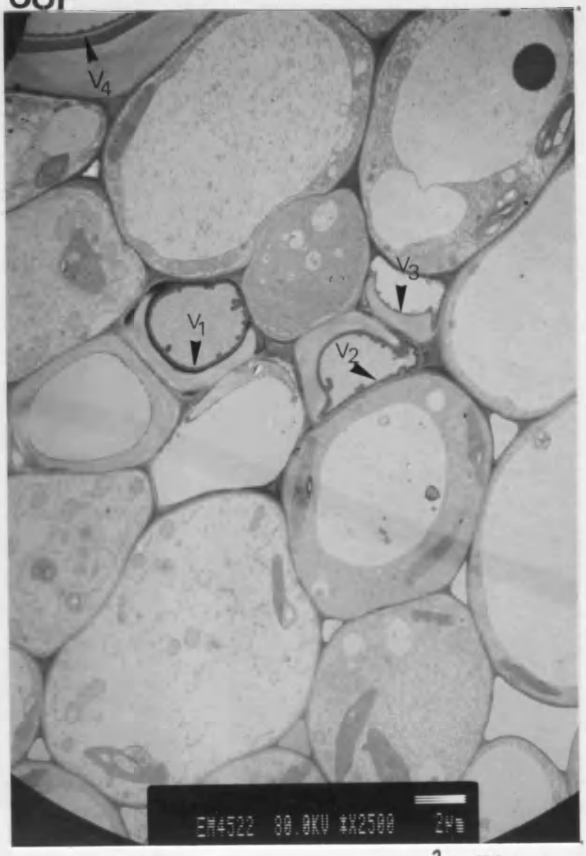
Plate 38G- Detailed view of a vessel with coating material lining the lumen (◀). Note the occlusion of the vessel-xylem parenchyma pit (⟶).

38E



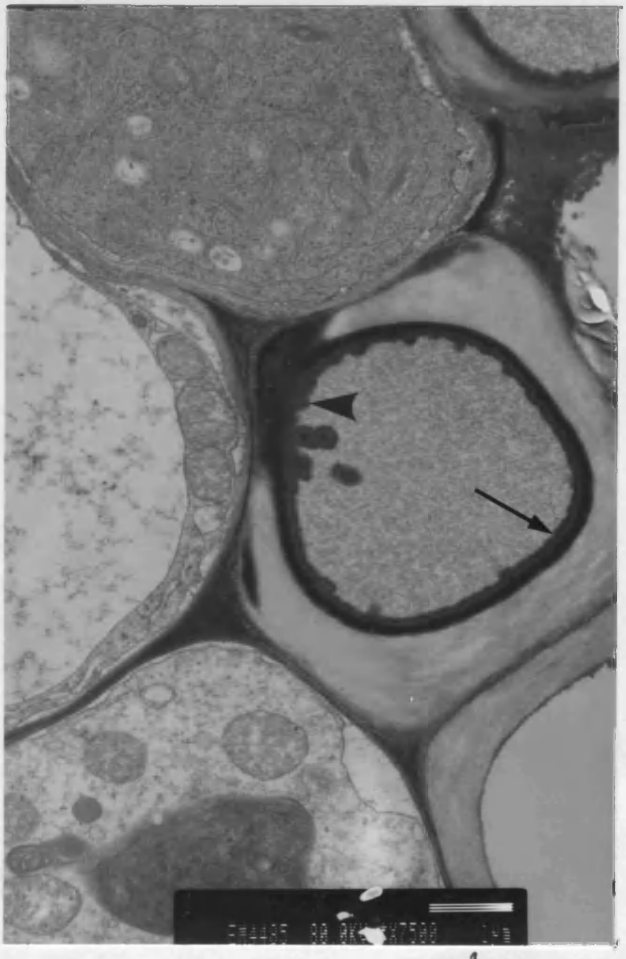
500 n m

38F



2 μ m

38G

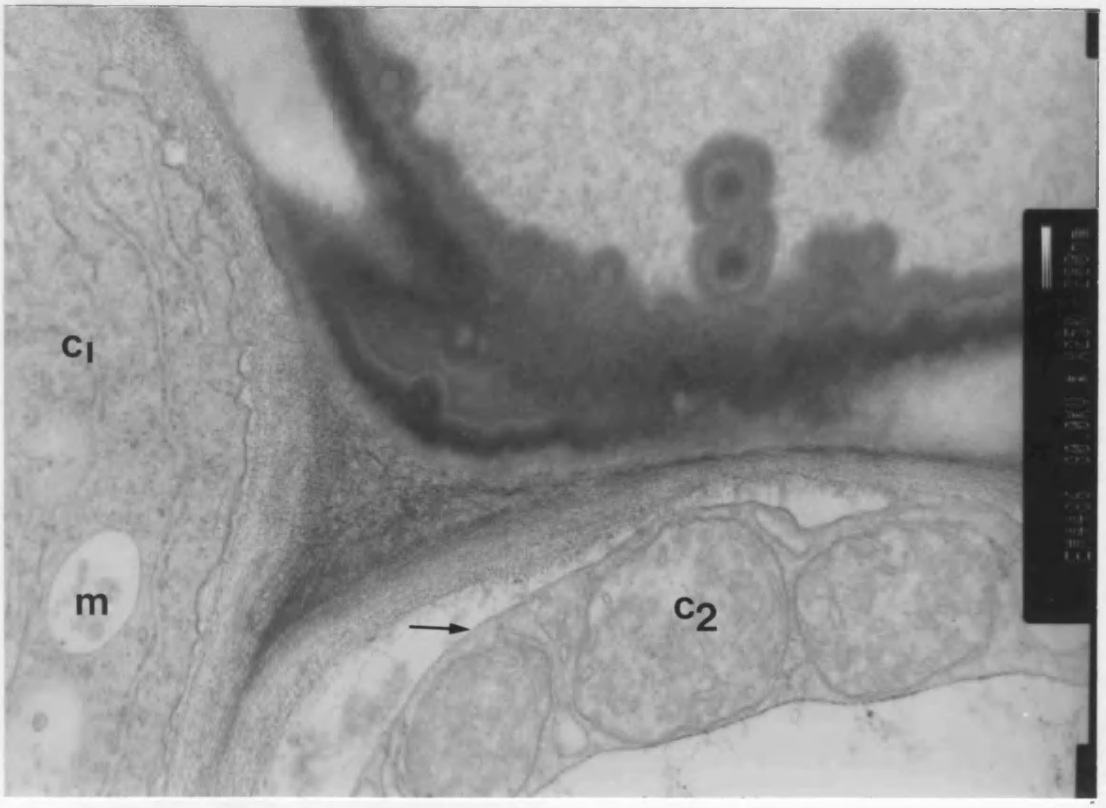


1 μm

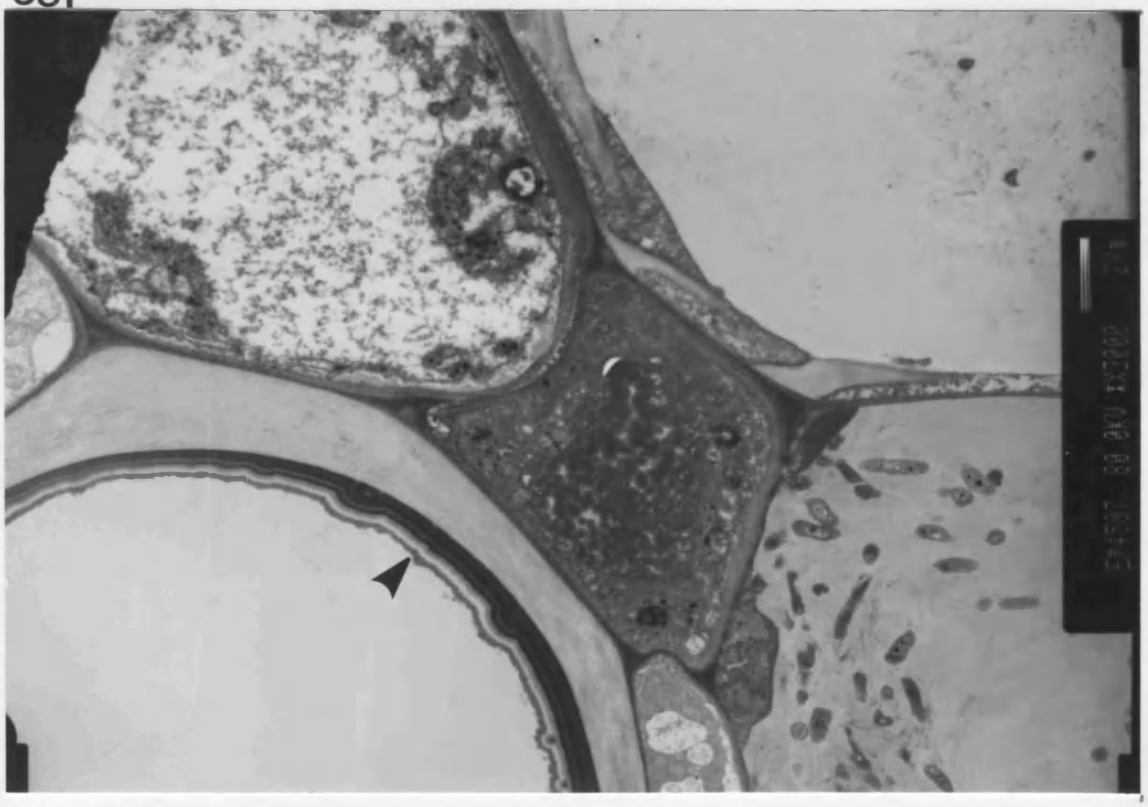
Plate 38H- High power of the pit area and vessel lining which appears to consist of successive layers. Adjacent C1 cell intact but cytoplasm of C2 is separating (⟶) from the cell wall. Note the swollen mitochondria (m).

Plate 38I- Xylem vessel with a layered lining (➤). Granular deposits fill pits of two xylem vessels and moribund xylem parenchyma cell.

38H



38I

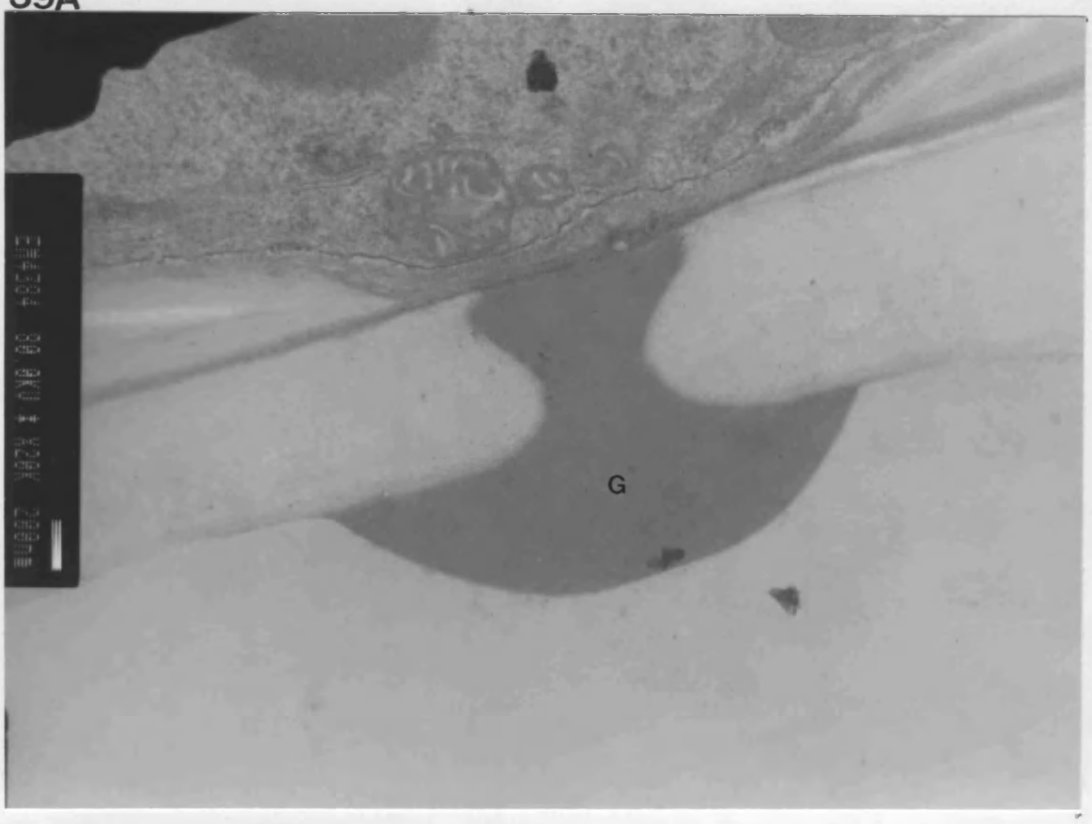


7.Host responses -Plates 39A and 39B- Stem

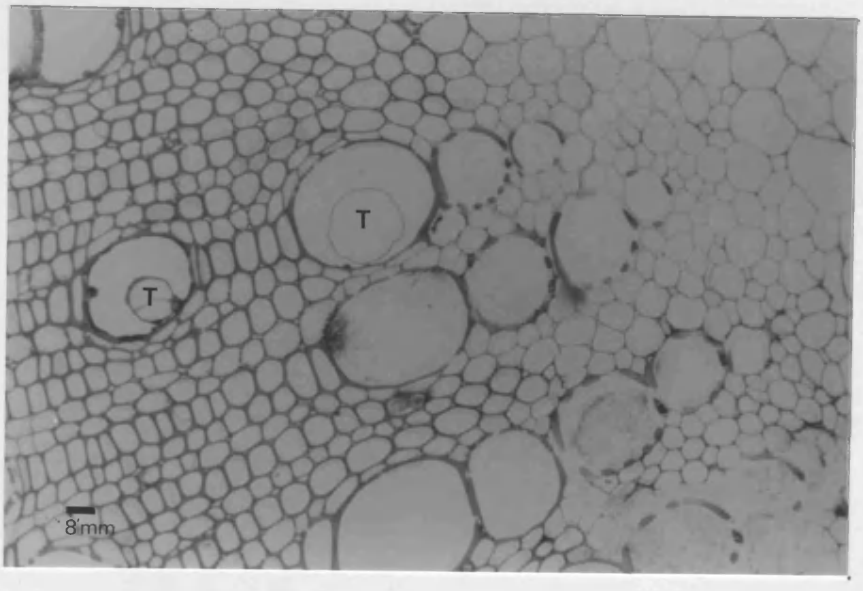
Plate 39A- Gel (G) apparently secreted by the xylem parenchyma cell through a pit into the adjacent vessel.

Plate 39B- Light micrograph of infected stem showing xylem tissue occluded with tyloses (t).

39A



39B



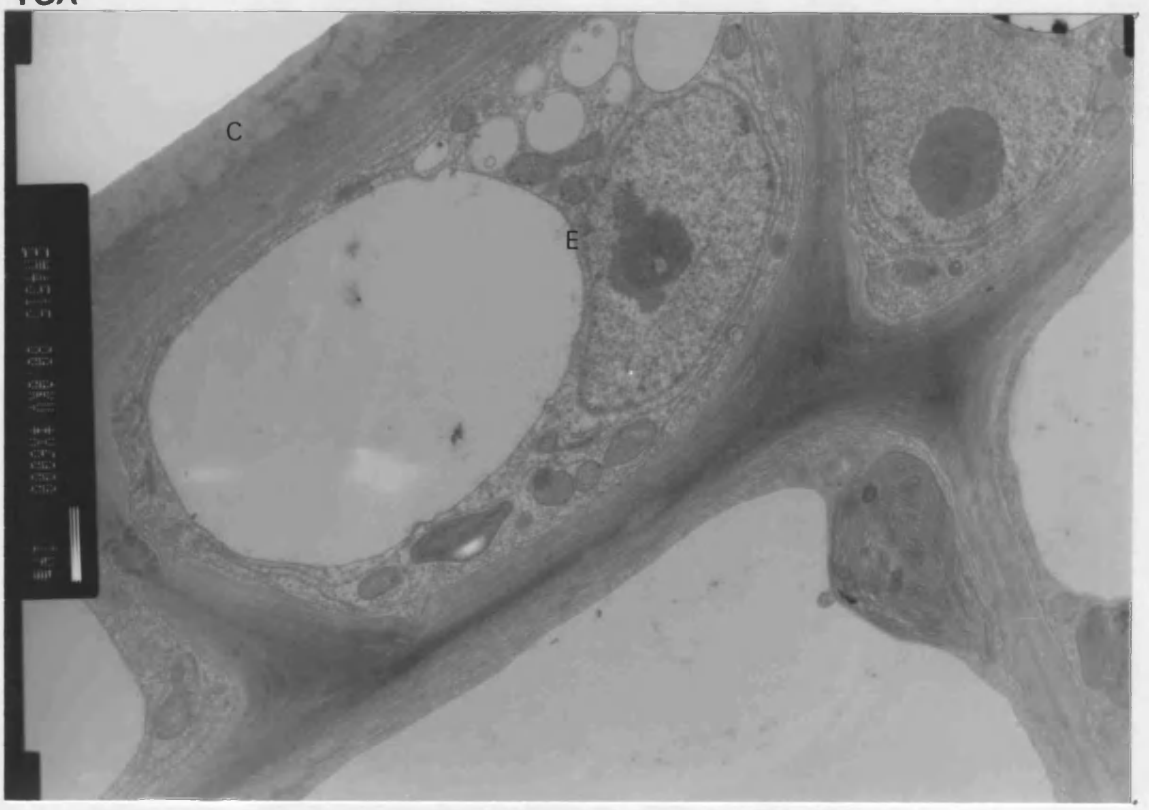
8.Effects on other cells- Plates 40A-40H

Plates 40A, 40B- Stem

Plate 40A- Epidermal cells (E) which appear to be intact and unaffected. Cuticle (C).

Plate 40B- Chlorenchyma cells also appear unaffected. Note that inspite of the large intercellular spaces (IS) no bacteria or deposits incurred by the disease were ever seen here.

40A



40B

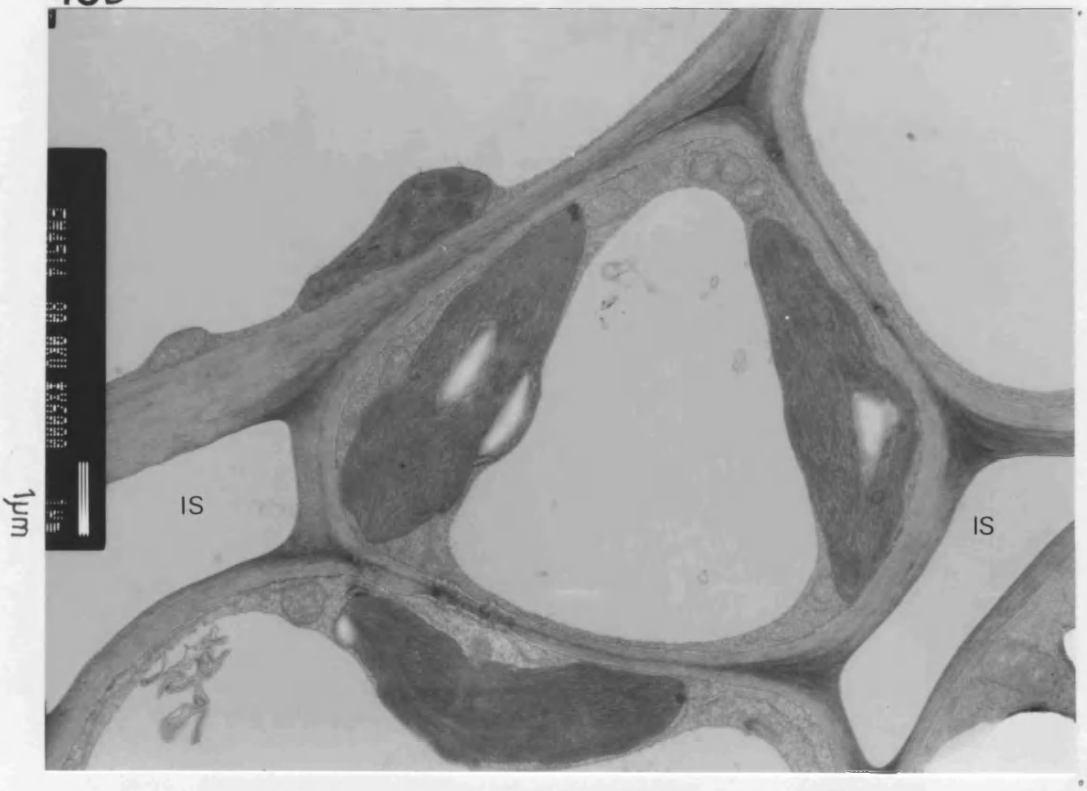
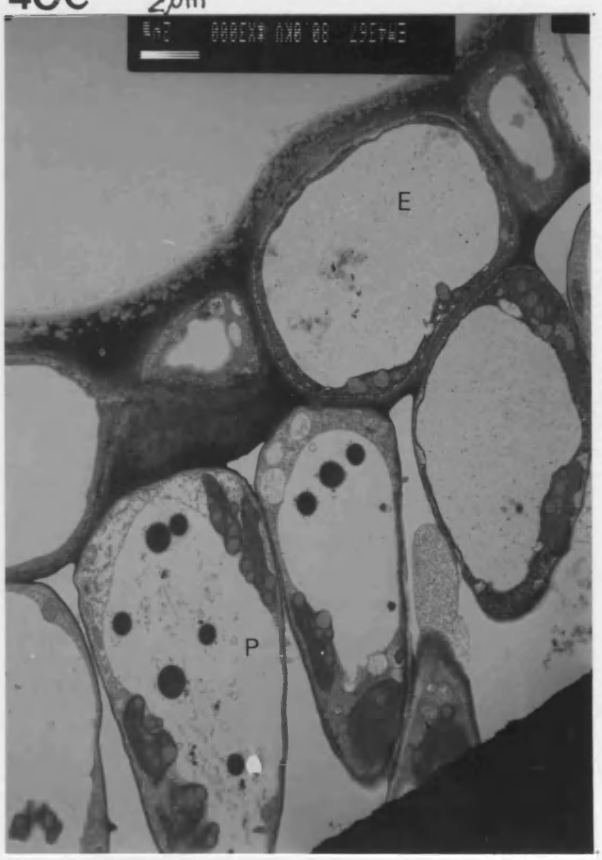


Plate 40C-40H- Leaf

Plate 40C- Epidermal (E) and pallisade (P) cells near an infected vein. Note dense globules in vacuoles of pallisade cells and chloroplasts.

Plat 40D- Lower power of infected single leaf vein and adjacent cells. Adjacent mesophyll and pallisade cells show advanced degeneration and cell walls though intact appear to be deformed.

40C



40D

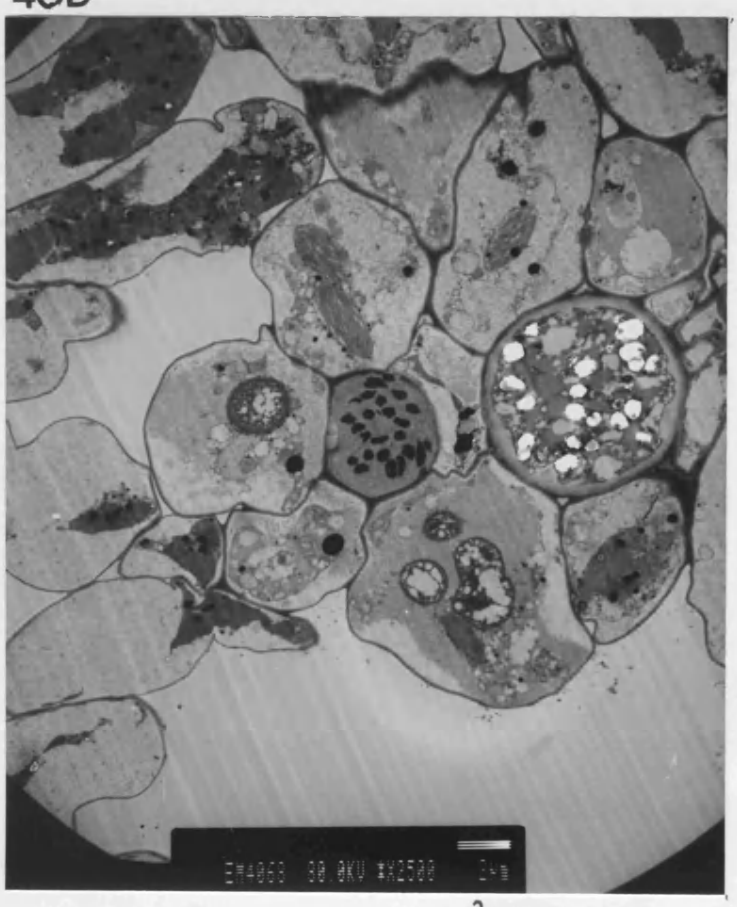
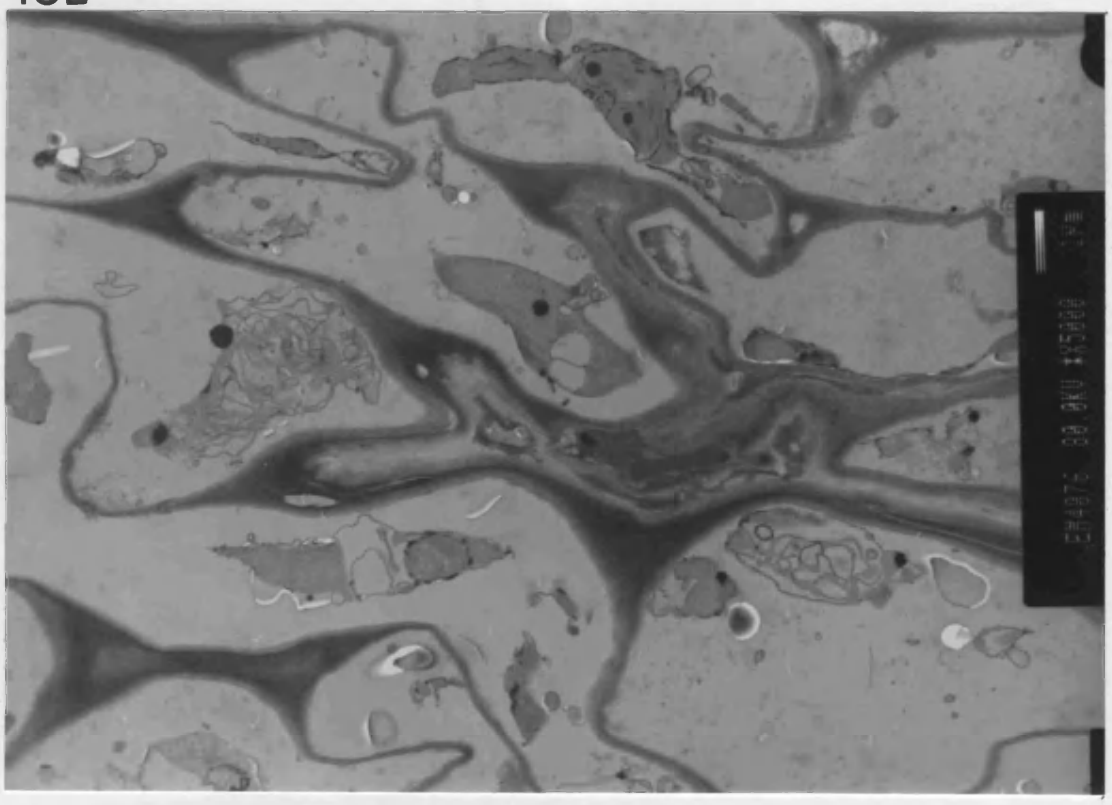


Plate 40E- High power of mesophyll cells of leaf tissue adjacent to an infected vein. All cells are in an advanced state of deformity with degenerate organelles.

Plates 40F - Localized areas of damaged cells. The infected vein is not visible or evident in these planes of sections.

40E



40F

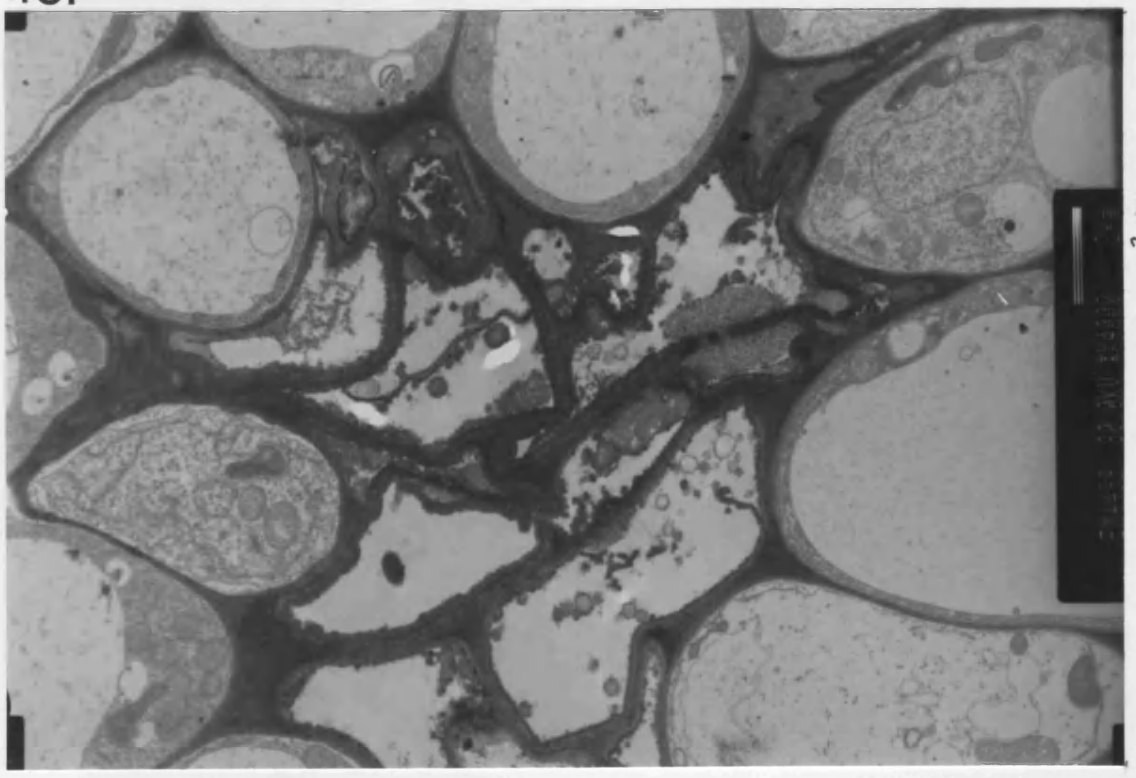
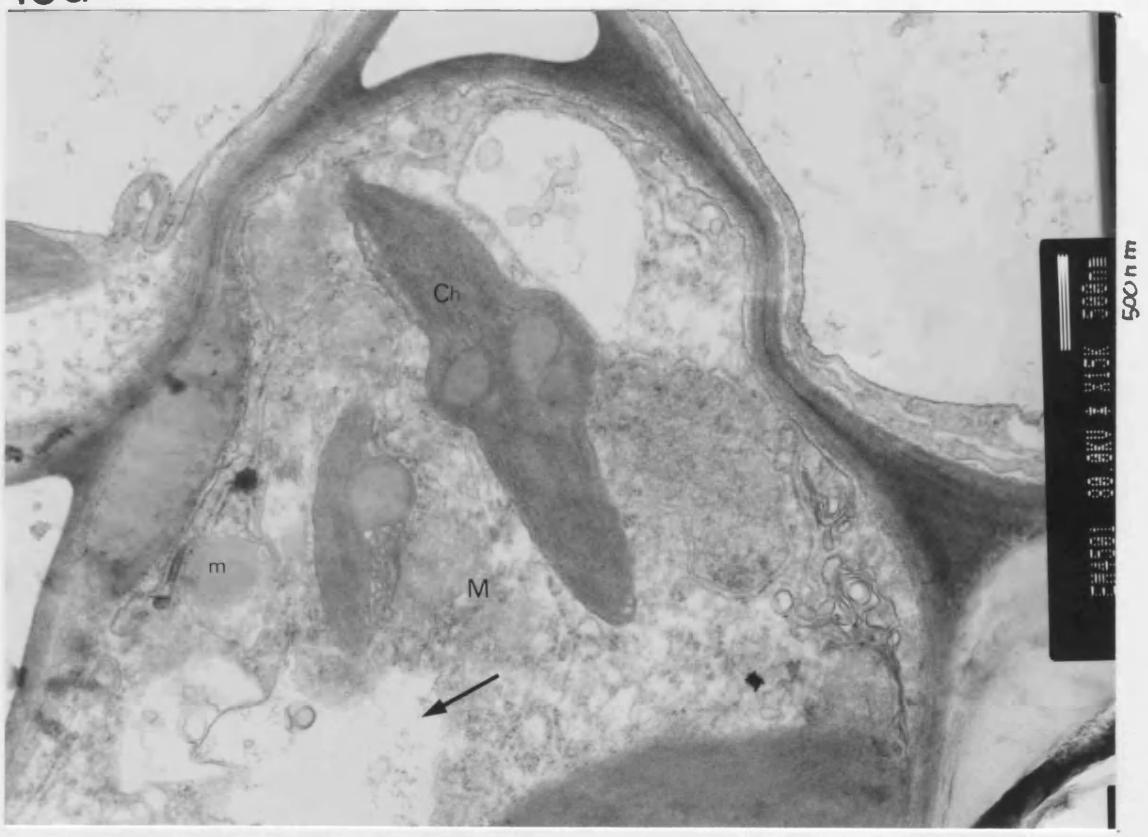


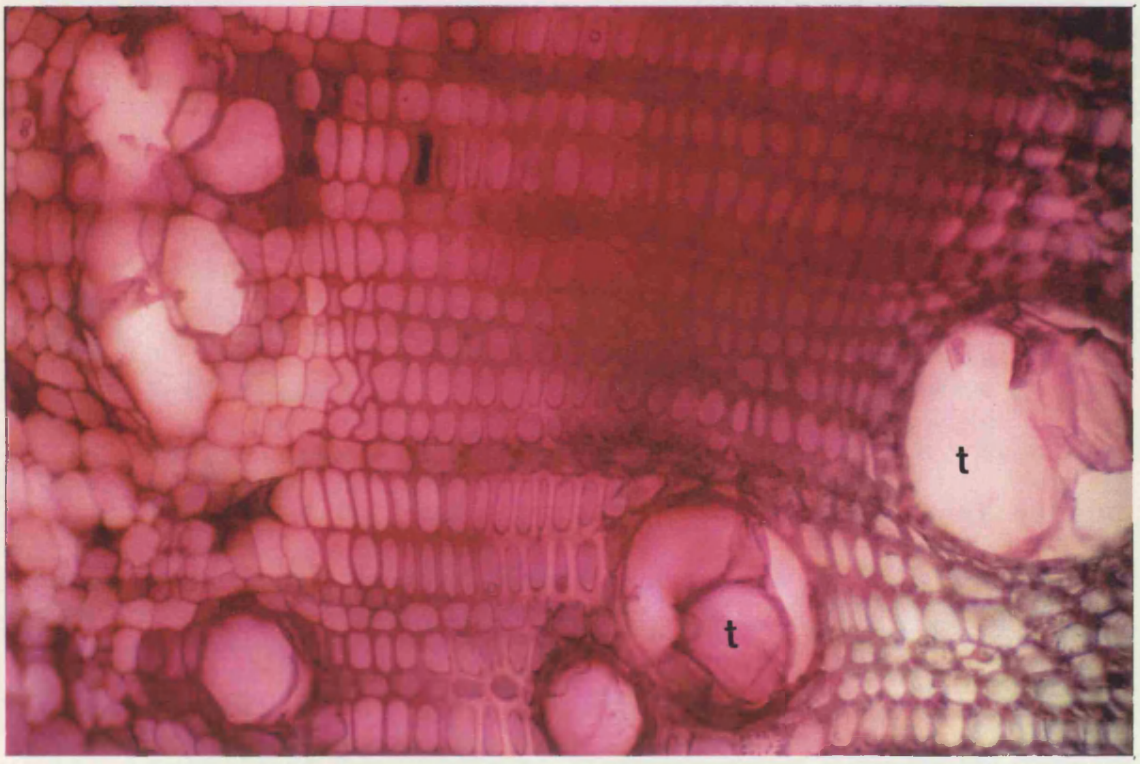
Plate 40G- Details of a mesophyll cell (M) adjacent to an infected vein (V). Shape and structure of chloroplasts is disrupted (Ch), mitochondria (m) are swollen and roundedand tonoplast is disrupted ⟷.

40G



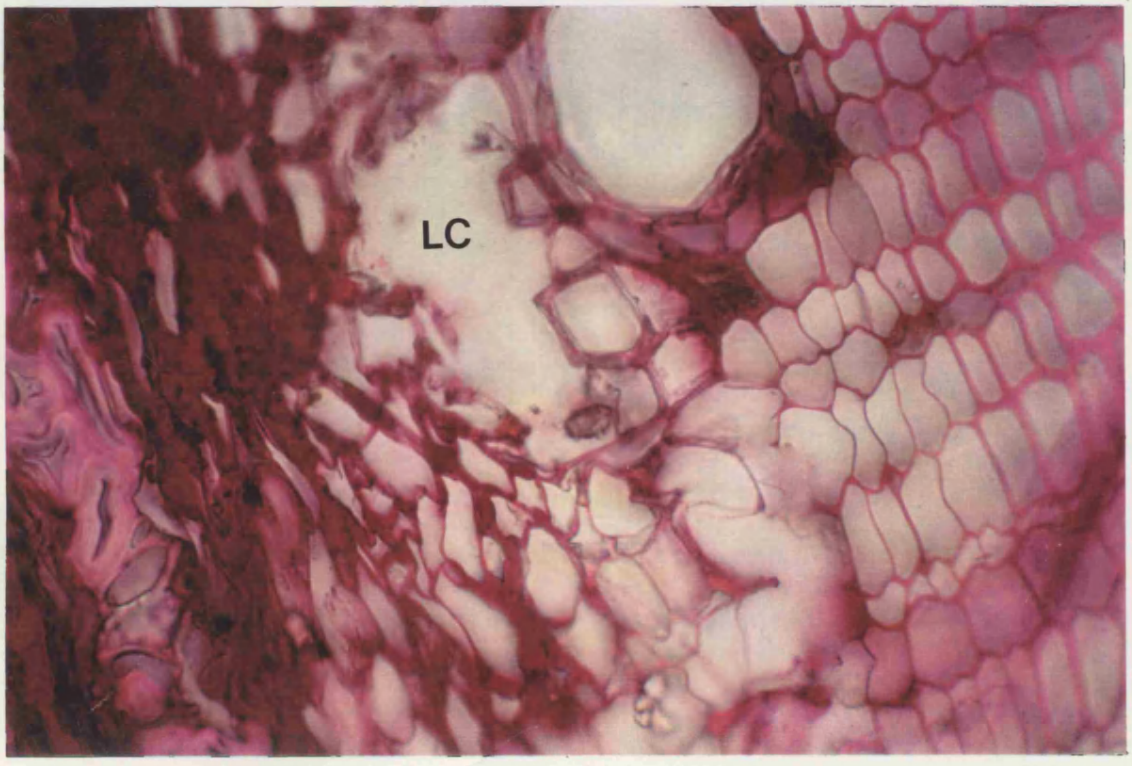
Plates 41A, 41B- Light micrograph of infected stem (20 days after inoculation) showing lysogenic cavities (LC) and tyloses (t).

41A



7μm

41B



7μm

DISCUSSION IV

Electron microscopy has enabled the micro-sites within which bacteria grow and respond to conditions within the intercellular environment to be clearly defined. For example, ultrastructural studies allow an examination of the role of pathogenicity determinants such as extracellular polysaccharide (EPS) and the activity of cell wall degrading enzymes and toxins during the early stages of infection development and also an assessment of the significance of the encapsulation and agglutination of bacteria on plant cell walls as mechanisms of resistance (Mansfield et al., in press). Coupled with physiological experiments, microscopy allows the timing and localisation of responses occurring within plant cells to be determined.

Xcm has been reported to gain entrance into cassava leaves through wounds or stomates (Lozano & Sequeira, 1974). Similarly vascular pathogens such as Corynebacterium michiganense and Pseudomonas solanacearum have been reported to enter their host plants through stomates (Nelson & Dickey, 1970). The bacteria were initially found in the stomatal openings and substomatal chambers and later were closely associated with the cell walls or were located in the intercellular spaces of collapsed parenchyma tissue. In contrast, although Xcm must have entered via stomates in leaves it was found

exclusively in xylem cells strongly suggesting its true adaptation as a vascular pathogen. Although the absence of Xcm in the intercellular spaces of leaves of the susceptible cassava cultivar tested cannot be explained, similar absence of bacteria in the intercellular spaces in the compatible interaction between X. campestris and cabbage have been observed. However, bacterial cells were present in the xylem conducting elements and intercellular spaces in the incompatible interaction (Bretschneider et al., 1989). Similarly, in an interaction between Pseudomonas glycinea and soybean, bacteria were seen in intercellular spaces only in the incompatible interaction (Drews et al., 1988).

Ikotun (1978) claimed that no bacteria were found in the lumen of xylem vessels but only within cells surrounding vessels thus he concluded that Xcm is not a vascular but a parenchymatous pathogen. This is in disagreement with the present work as well as with the findings of Perreaux & Maraite (1978) in a histopathological study using fluoresent microscopy on cassava stems infected with Xcm. They also reported migration of bacteria through xylem vessels but eventual movement into intercellular spaces of xylem parenchyma.

Xcm is reported to enter the xylem tissues in the stem either by spreading through the petiole from infected leaves or by planting infected cuttings (Lozano,

1975). The former method of spread was not evident in the present study as inoculated leaves abscissed after the bacterium spread down the petiole (Section II, 3i). It seems likely that the pathway for bacterial migration through host tissues can be influenced by the method of artificial inoculation and sampling, and care should be exercised in interpretation of experimental results. The method used to inoculate Xcm into the stem in the present study was deliberately chosen in order to introduce the bacterium into most cell types of the stem. The presence of Xcm only in vascular tissue at the time of sampling (12 days after inoculation) again indicates its true adaptation to colonize xylem tissue.

Vessel-to-vessel spread is dependent on the ability of the pathogen to traverse the primary walls at pit 'membranes'. Different pathogens appear to have varying ability to break down the pit membrane walls (Wallis et al., 1973; Wallis & Truter, 1978), but most wilt pathogens that have been investigated ultrastructurally appear to be able to dissolve the pit walls sufficiently to allow movement of bacteria from vessel to vessel (Van Alfen, 1982). In most cases, this movement of bacteria appears to be restricted initially to the vessels (Suhayda & Goodman, 1981; Wallis & Truter, 1978; Wallis et al., 1973; Wainwright & Nelson, 1972; Bretschneider, 1989). Wallis (1977) reported that the primary wall of

parenchyma cells abutting that of a vessel's pit membrane is less affected by C. michiganense pv michiganense than are the pit membranes of adjacent vessels. If the parenchyma walls abutting pit membranes are more resistant to pathogen factors than vessel-vessel pit membranes, the bacteria would tend to be restricted to vessels. Such initial restriction to the vessels would not be a handicap to the spread of the pathogen since there are fewer walls for the pathogen to breach as it moves in the vessels than in other tissues. However, vascular wilt bacteria are reported to have the ability to invade initially through intercellular spaces (Chërif et al., 1991) before subsequent colonisation of xylem vessels.

The speed of the spread of Xcm in cassava tissues also indicated vascular movement (Section II,3i). Wainwright & Nelson (1972) report of X. pelargonii movement in infected Pelargonium spp. in xylem vessel elements and subsequent lateral movement into xylem parenchyma cells. Similarly, X.campestris pv campestris cells were found within xylem conducting elements of infected cabbage leaves accompanied by a dense fibrillar material which appeared to occlude the entire lumen of the cells (Wallis et al., 1973; Bretschneider et al., 1989).

The nutrient content of xylem fluid from a number of

plants has been found to be low (Coplin et al., 1974; Singh & Smalley, 1969). If, however, the bacterial cells are continually washed with the nutrient fluid, which they will be initially, it might be sufficient for growth. Also cell walls may provide a nutrient source, but any form of cell wall breakdown was not observed here although the Xcm isolate used was shown to be capable of producing high levels of the cell wall degrading enzyme PGL in vitro (Section III). The lack of host cell wall breakdown may reflect a very low production or an absence of pectolytic activity in vivo which was also evident in extracts from infected cassava tissues (Section III,5). Or it may be that the levels produced may be only sufficient to degrade the primary walls of xylem pits at the early stages of growth. Infected cassava stems at later stages of symptom production showed lytic pockets in the xylem tissues which could result from the breakdown of cell walls. Therefore, it could be postulated that higher levels of cell wall degrading (eg. pectolytic) enzymes may be synthesised towards the latter part of the disease cycle when the bacterial numbers would be higher and also when any host resistance mechanisms may have been suppressed. There is no direct evidence for this because the preparation of host material for TEM at this stage could not be done as the tissues were too fragile to undergo the process. However, an alternative mechanism might be provided by Goodman &

White (1981) who described the development by Erwinia amylovora of lysogenic cavities in apple and pear tissue which were filled with bacteria. They hypothesised that xylem vessel rupture, releasing bacteria, is due to the loss of turgor and subsequent collapse of xylem parenchyma cells (which were plasmolysed as the pathogen was unable to degrade cell walls enzymically. In marked contrast to this study, Ikotun (1978) reported extensive enzymic degradation of host cell walls. A delay of cell wall breakdown similar to that shown in the present study was observed in tomato roots infected with P. solanacearum (Wallis & Truter, 1978). Similarly, P. solanacearum has been reported not to affect the morphology of vessel walls until bacterial numbers are high (Wallis & Truter, 1978); however, X. campestris pv campestris causes cell wall dissolution early in the infection process (Wallis et al., 1973).

Xcm cells colonised xylem parenchyma cells adjacent to vessels and these could have provided nutrients necessary for bacterial growth. The adjacent parenchyma cells have been assumed to be a major source of nutrients for vascular pathogens restricted to xylem vessels although the method of nutrient supply must follow damage to membrane permeability with resulting leakage into vessels (Wallis et al., 1973). This has been supported by reports from Coplin et al. (1974) and Bhushan & Prasad (1987) that amino acid content of xylem fluid of

infected plants is higher than that from healthy plants.

Symptom induction

Copious amounts of fibrillar material were seen to surround the bacterial cells in xylem tissue and intercellular spaces in infected cassava leaves and stems. This material could represent bacterial EPS as the structure is similar to that of bacterial EPS as reported by Harper et al. (1987), Brown & Mansfield (1988), Bretschneider et al. (1989) & Braun (1990). Bacterial EPS has been reported to play an important role in the physical blockage of vessels by plugging the pit membranes as macromolecules cannot pass through pit membranes (Van Alfen, 1982; Braun, 1982; Chatterjee & Vidaver, 1986). Very low levels (picomole quantities) of the high molecular weight (ca. $2x10^6$ daltons) EPS produced by Clavibacter michiganense subsp. insidiosum was sufficient to completely plug the water-conducting system of alfalfa (Van Alfen et al., 1983). Physical blockage of vessel lumens and of pits with EPS was clearly demonstrated in the present study and this could be a major factor that contributes towards the wilting of infected cassava plants.

EPS appears to consist of alginates in the case of pseudomonads eg. P. syringae pvs. coronafaciens, tabaci and phaseolicola and of xanthan in the case of

xanthomonads eg. X. campestris pvs. glycines, pelargonii and citri (Rudolph et al., 1989). The repeating unit of xanthan gum is a penta-saccharide composed of two glucose, one galacturonic acid and two mannose moieties and is a very large molecule (Coplin & Cook, 1990).

Another way that EPS may be critical for the virulence of wilt bacteria is to facilitate their movement within and out of infected plants (Van Alfen, 1982). Bacteria within xylem vessels and intercellular spaces in a matrix of EPS, fill the spaces available and could exert physical pressure on the pit membrane by the expansion of the matrix by absorbing water. This may facilitate the movement of bacteria en masse through the vessels of infected plants by damaging the pit membranes (Schouten, 1988). Several electron-micrographs in the present study indicate the possibility of the exertion of physical pressure on pit membranes by bacterial cells and EPS that surrounded them.

Another role attributed to EPS is the prevention of binding of the bacterium to host cell walls (Sequiera et al., 1977; Sequiera & Graham, 1977). Presumably, the EPS acts either to saturate these binding sites or to prevent exposure of bacterial receptor molecules. In either case the EPS would prevent the binding and thus contribute to the virulence of the bacterium by counteracting a host

defence mechanism.

EPS has been reported to be associated with other bacteria known to be plant pathogens. Some of these interactions are soybean leaves infected with X. campestris pv glycines (Jones & Fett, 1985), bean leaves and pods infected with P. syringae pv phaseolicola (Harper et al., 1987; Brown & Mansfield, 1988), cabbage leaves infected with X. campestris pv campestris (Wallis et al., 1973; Bretschneider et al., 1989), sweet orange leaves infected with a xylem limited bacterium similar to Xylella fastidiosa (Chagas et al., 1992), maize inoculated with Erwinia stewartii (Braun, 1990) and rice inoculated with X. campestris pv oryzae (Wakimoto 1985).

However, whether wilting ability is purely a function of EPS size and viscosity is debatable, since very little is known about how the structure of EPS influences pathogenicity (Coplin & Cook, 1990). EPS- mutants of X. campestris pv campestris have been shown to have reduced virulence (Ramirez et al., 1988; Sutton & Williams, 1970; Whitfield et al., 1981). However, in a few instances EPS- mutants have retained partial to full virulence (Barrere et al., 1986; Coplin & Majerczak, 1990; Xu et al., 1990).

According to Ikotun (1978), the wilting due to blockage of water flow in Xcm infected cassava plants is

not caused by the physical blockage of xylem vessels but by the loss of structural integrity of cells along the vessels that contain bacteria. The present work is in complete disagreement with these observations as large numbers of bacteria and bacterial EPS completely occluded the lumens of vessels and tissue integrity was not lost.

The electron lucent area that was sometimes observed around Xcm cells embedded in acidic polysaccharide has been observed in other similar bacterial/plant interactions (Brown & Mansfield, 1988; Bretschneider et al., 1989). This may be caused by shrinkage of EPS during preparation for microscopy, or may represent the location of lipopolysaccharide (LPS).

However, Wallis (1977) reported that xylem vessels of tomato plants infected with Corynebacterium michganense showed insufficient amounts of vessel plugging material or structures to account for the wilting of plants. It was apparent that the wilting of the host was due to degradation of the conducting tissue by bacterial enzyme action rather than blockage of the xylem vessels by bacterial EPS. However, this observation is open to criticism because (i)no attempt to fix EPS for TEM eg. with ruthenium red (Mansfield et al., 1992) and (ii). as only very low levels of EPS are sufficient for blockage of vessels (Van Alfen, 1983).

A study of the pathogenicity and multiplication patterns of EPS$^-$ mutants of Xcm would confirm the role played by EPS in pathogenesis of cassava.

The electron dense deposits which were very abundant on cell walls of xylem tissue in both cassava stems and leaves is established as a common feature in plants infected by vascular pathogens (Cooper, 1981). In the present study, their appearance did not correspond strictly to the four distinct types of vessel coating viz. smooth, 'bubbly', irregular and fibrillar found in wilted leaves of chrysanthemums infected with Verticillium dahliae as described by Robb et al. (1979). The coating or deposits ranged from smooth to irregular and therefore any attempt to classify the separate types on this basis seems artificial.

Several workers have reported that the coating material had a high phenol content (Favali et al., 1978; Nemec, 1975; Robb et al., 1978) but Robb et al. (1979; 1982) suggested that phenolic compounds were confined to the fibrillar material and further claimed that the bulk of the coating material is lipid with little or no carbohydrate content. As most Xanthomonas spp. are reported to be lipolytic (Wainwright & Nelson, 1972), it is feasible that these lipids could be the result of enzyme activity on adjacent xylem parenchyma cells. However, Robb & Busch (1982) observed that vessel coating

material similar to those observed in plants infected with Vericillium dahliae appeared along the walls of water stressed chrysanthemum and sunflower plants. Therefore, they suggested that this response is a generalised plant response to wounding.

Chërif et al. (1991) report of a typical reaction feature observed in cucumber tissues infected with Pythium ultimum as a deposition of thick layers of an opaque osmiophilic material, along secondary thickenings and pit cavities of xylem vessels. They assumed that this deposited material is of plant origin since the coating was frequently observed in advance of xylem vessel colonization. They also concluded that the amorphous texture of this material, as well as its high electron density, suggests that the material is enriched with phenolic compounds.

Wainwright & Nelson (1972) also observed similar deposits on vessel elements in Pelargonium plants infected with X. pelargonii, which appeared as a rough layer constricting the lumen. This material gave a positive reaction to lignin. Such wall deposits have been also observed in interactions between the vascular fungal pathogens Fusarium oxysporum f. sp. vasinfectum and cotton plants (Shi et al., 1991) and F. oxysporum f. sp. lycopersici or Verticillium alboatrum in resistant and susceptible tomato (Bishop & Cooper, 1984).

Wallis et al. (1973) suggest that the irregularly shaped electron opaque granules observed in the lumina of vessels of cabbage invaded by X. campestris comprises a lignin component of the vessel wall which could be released following bacterial enzyme action on the primary walls of the invaded vessels. However, Fett & Jones (1985) observed similar electron dense deposits on cell walls of soybean infected with incompatible strains of X. glycines which might suggest a means of immobilisation of bacterial cells. Without chemical or cytological analysis interpretations will remain highly speculative.

However, in this context some success has been achieved. Mazau & Tugayë (1986) claimed similar deposits composed of a substance different from all previous reports. According to their work, fungal, bacterial and viral infections triggered accumulation of hydroxy proline rich glycoproteins (HRGP) on host cell walls. They claimed that this response occurred in relation to resistant as well as susceptible reactions. However, Mansfield et al. (1992) identified as HRGPs material that encapsulated bacteria and found that the accumulation of this material encapsulating cells of P. fluorescens was consistent with their involvement in agglutination and immobilization of saprophytic bacteria on plant cell walls. Immunocytochemical studies showed that the major accumulation of HRGPs occurs in live cells adjacent to those undergoing HR. The composition of the extensive

electron opaque material deposited on cassava cell walls remains to be identified.

The dense material which accumulated on the cell walls of xylem tissues may prevent entry of water and nutrients into living cells from adjacent invaded vessels, thereby eventually causing their death. This could explain the localised death (necrosis) of cells in cassava leaves adjacent to infected vessels. In addition blockage of vessels by bacteria, bacterial EPS and host products would lead to wilting and ultimate death of the host plant. Other evidence has also suggested that vessel coating material (Cooper, 1974; Wallis & Truter, 1978; Robb et al., 1979; 1980; MacHardy, 1976; Pegg et al., 1976) and infusion of these structures by phenolic compounds (Robb et al., 1978) may inhibit lateral water flow.

In infected cassava tissue, xylem parenchyma cells adjacent to infected vessels developed a thicker 'protective layer' on the inside of the cell wall. A similar structure has been observed in chrysanthemum xylem parenchyma cells infected with Verticillium dahliae (Robb et al., 1979); this layer was shown to contain phenolic compounds and these could render the walls impervious to water and it would as suggested earlier, prevent lateral redistribution of water through walls of xylem parenchyma cells. This layer could also be assumed

to be produced to protect the xylem parenchyma cells from deleterious conditions within adjacent infected xylem vessels. Mueller & Beckman (1984) also reported of a distinctive wall deposit occurring on mature 'contact cells' (xylem parenchyma cells) in the metaxylem of tomato stems. This was similar to the layers deposited in response to vascular disease and was presumed to be involved in the control of the movement of water and solutes in and out of the vascular elements.

Light microscopical studies of infected stem sections of cassava showed occlusion of xylem vessels by tyloses. Tylosis following infection by wilt pathogens has been described in many previous studies (Beckman et al., 1972; Dixon & Pegg, 1969; Elgersma, 1973; Robb et al., 1979; Wallis & Truter, 1978; Wainwright & Nelson, 1972). Most information relating to the ultrastructure of tyloses has been centred around the role played by the xylem parenchyma cell pit membrane during tylose development. Pertaining to this are two viewpoints: that the tylose wall is (Beckman, 1971; MacDonald & McNabb, 1974) or is not (Meyer, 1967; Meyer & Côtë, 1968) derived from the xylem parenchyma cell pit membrane. Observations of tyloses in the present study were not detailed enough to conclude this clearly.

The mechanisms of induction of tyloses remains unclear. Involvement of cell wall degrading enzymes

produced by the pathogen or higher levels of IAA incited by the pathogen have been suggested as possibilities (Wallis & Truter, 1978).

Apparently bacteria may enter a preformed tylose at any stage of its development. Tylosis may play an important role by occlusion of vessels in preventing spread of bacteria. On the other hand, a sudden release of large numbers of bacteria from disrupted tyloses may cause rapid and successful colonization of the xylem vessels (Wallis & Truter, 1978). However, the present study did not show any bacteria associated with tyloses and thus any assumptions of this nature could not be made.

Vessel occlusion by gels although not frequently observed in the present study, is a common feature of infected vascular tissue (Braun, 1990; Mollenhauer & Hopkins, 1976; Bishop & Cooper, 1983) and may be a general plant response to stress. Gels appear to be largely pectinaceous in character (Beckman, 1966) although gels containing lignin and callose have been reported (Palmieri et al., 1979). Gels may also be stabilised by phenolic compounds (Robb et al., 1979). Gels and tyloses may themselves contribute to the development of disease symptoms by reducing water movement in the vessels while simultaneously restricting the internal spread of the bacteria . Beckman (1966) has

proposed that localization of infection by vascular exclusion is a general mechanism of resistance to vascular infections. This hypothesis of forming tyloses and gels to infer resistance by localization of the pathogen should be confimed by a parallel study of a resistant cassava cultivar inoculated by Xcm.

Only xylem cells containing Xcm and some cells adjacent to them were damaged in both stems and leaves but the cells distal to these remained apparently undamaged. This provides further tentative evidence that there is little likelihood of a diffusible toxin being involved in disease induction.

It would appear that symptoms of water stress in cassava plants infected with Xcm are due to a combination of several factors. These include: the presence of large amounts of bacterial EPS which usually completely filled infected vessels and the presence of bacterial cells in vessels; the blocking action of tyloses and gels; the deposition of an electron dense material on pit membranes and cell walls which could prevent movement of water and nutrients between vessels and from vessels to cells, also bacterial pectate lyase may contribute by release of high molecular weight fragments from primary walls of pit membranes.

GENERAL DISCUSSION

Studies done both in vitro and in vivo confirmed previous reports (Lozano & Sequeira, 1974a;1974b Williams et al., 1973; Lozano, 1975; 1986; Lozano & Booth, 1975) on the pathogenicity of Xcm on cassava. Previous reports on the disease seemed to indicate two distinct stages under natural conditions ie. leaf blight and systemic invasion. Different methods of inoculation used in this study produced disease symptoms similar to these phases and vascular invasion in leaves resulted from leaf lesions.

Plantlets and suspension cultures proved to be suitable tissue culture systems for pathogenicity studies of the bacterium. The interaction between Xcm and suspension cultured cells of a susceptible cassava cultivar indicated that a high molecular weight factor could be responsible for cell mortality caused by Xcm. However, the fluids from these inoculated suspension culture systems failed to show any toxicity towards cassava. More detailed studies of the factor causing cell death in the suspension culture system and ultrastructural studies of the interaction should give a better understanding of the system. In particular the involvement of pectate lyase (see below) should be addressed as this was the only factor produced under realistic culture conditions which showed toxicity to

cassava cells. Cassava lines resistant to Xcm could be selected by regeneration of plants that would survive coculture with the bacterium. However, as regeneration of plants from suspension cultured cells could not be acieved this method could not be used.

Resistance mechanisms of cassava to Xcm could not be investigated as the field resistant cultivars available did not show a clear cut resistance but only apparent tolerance to the bacterium. Polygenically controlled field resistance is often sensitive to environmental conditions. The importance of environment in the expression and selection of polygenic resistance has been demonstrated with bean infected with X. campestris pv phaseoli (Webster et al., 1983) and cotton infected with X. campestris pv malvacearum (Arnold & Brown, 1968). There is a clear need for a better understanding of resistance as cassava bacterial blight is a key disease of this major crop. Very few resistant lines of cassava are available and although some were shown to be resistant in both glass-house and field conditions in Colombia (CIAT, 1975), similar inoculation methods did not show resistance in the present study. Therefore these resistant cultivars may be of limited value as the resistance might not be sufficiently expressed in other regions or might be overcome by Xcm isolates from other regions. Therefore more sources of resistance are needed and also pathogen variability should be tested. In vitro

plantlets could be perhaps used as host material in these tests as they would provide an easy screening system.

Systemic movement of Xcm in cassava plants was suggested here by the rate of spread of the bacterium in stems and leaves. Ikotun (1978) claimed Xcm to be a 'parenchymatous' pathogen but transmission electron microscopy in this study showed the bacterium to be present in xylem tissue. Lozano & Sequeira (1974a), Lozano (1975), Maraite & Meyer (1975;1978) also reported of vascular movement of Xcm in cassava. The presence of most features associated with vascular pathogenesis such as tylosis, gels, electron-dense deposits on cell walls and protective linings in infected cells further suggests the systemic nature of the pathogen. TEM studies of resistant cultivars of cassava infected with Xcm should indicate whether these responses were a reflection of susceptibility or the beginnings of resistance reactions. Vascular pathogenesis could play an important role in the spread of the disease as the main propagation method of cassava is planting stem stakes.

Bacterial movement between xylem vessels could be facilitated by two factors which may act individually or in combination. These are namely, bacterial EPS and pathogen produced cell wall degrading enzymes.

Large amounts of EPS produced by Xcm were observed

to occlude xylem vessels. EPS could absorb water to become hydrated and exert a swelling pressure such as demonstrated with EPS of E. amylovora (Schouten, 1988), which could help to physically push through pits carrying bacterial cells in the process. Some electron micrographs showed such streaming of EPS across disrupted pit membranes. The occlusion of xylem vessels with bacterial cells and EPS and the resulting reduction of water supply in the plant is likely to be the major factor in wilting. Xanthomonas campestris has been reported to produce copious amounts of EPS known as xanthan (Harding et al., 1987; Sutton & Williams, 1969; Coplin & Cook, 1990; Rudolph et al., 1989). The chemical structure of xanthan has been studied and shown to be a repeating unit of pentasaccharide composed of two glucose, one galacturonic acid and two mannose moieties (Coplin & Cook, 1990). Mansfield et al. (1992) have raised an antiserum to Xanthomonas campestris pv vesicatoria which contains components that were recognised by antigens in EPS produced by the bacterium. They claimed that it allowed differentiation between plant and bacterial components. This could be useful in recognition of bacterial EPS in Xcm infected cassava plants. Tests with EPS$^{(-)}$ mutants of Xcm should be carried out to further clarify the role of EPS in pathogenesis. These EPS$^{(-)}$ mutants should be analysed chemically to show whether they still produce small amounts of EPS or tested

immunologically (see above) to show if they produce EPS *in planta* but not *in vitro*. The amount of EPS required for symptom production and the features of EPS eg. viscosity, size) important for pathogenicity should also be investigated.

Pathogenicity factor(s) other than EPS such as PGL or a toxin are also likely to be involved because suspension cultured cells of cassava are killed by the bacterium and also because *Xcm* causes ion leakage from cassava discs.

However, there was no indication of high levels of methionine required for toxin production being present *in planta* unless bound methionine is released by proteolytic activity of *Xcm*. Nevertheless, cassava is noted for its low overall (free and bound) methionine content. Also, TEM studies did not show membrane damage to cells distal from infected cells to indicate possible involvement of a diffusible toxin.

With regard to pathogen produced cell wall degrading enzymes, highest production of the pectolytic enzyme PGL was detected in *Xcm* culture fluids with cassava cell walls as carbon source. The overall pattern of production indicated the inducibility of the enzyme; the conditions might mimic those within xylem vessels ie. availability of primary cell walls (at pits) under non-repressive conditions. The culture fluid macerated cassava tissue

and caused mortality of suspension cultured host cells indicating endo action of PGL. However, extracts from infected cassava plants did not show any PGL activity but this may be due to inactivation of the enzyme eg. by plant phenolics. Very low levels of PGL were toxic to cassava cells and could be sufficient to weaken primary walls in in xylem vessels or in xylem parenchyma cells to facilitate entry into cells. Low levels of PGL may also be sufficient for dissolution of pit membrane which would facilitate movement along and between xylem vessels. These observations contradict those of Ikotun (1984a) who has reported massive cell wall degradation in cassava and the production of pectinmethylesterase (PME), but this enzyme was not observed in the present study. Alternatively, light micrographs of cassava stems in an advanced state of disease showed lytic cavities in the vascular region which could have been caused by high levels of PGL produced at this stage. However, these cavities could be formed by 'multiplication pressure' caused by continued division of bacteria as hypothesised by Green (1972). TEM studies of plants with advanced disease symptoms was not undertaken because the tissue was too fragile at this stage for processing for TEM..

As iso electrofocusing showed only one form of PGL, it should be relatively simple to inactivate genes coding for this single form to acquire mutants without PGL

activity. This would provide a better understanding of the role of the enzyme. This approach has already proved successful in a number of systems by using Tn5 mutagenesis and subsequent marker exchange such as with Xanthomonas campestris pv campestris (Dow et al., 1989), X. campestris pv vesicatoria (Beaulieu et al., 1991), E. chrysanthemi (Daniels et al., 1984; Reid & Collmer, 1988; Roeder & Collmer, 1985).

Tests for toxicity of the purified enzyme would also be required for the results to be more conclusive but culture fluids of the bacterium grown in the same medium but with glucose as carbon source rather than cell walls contain no PGL activity and should have indicated the presence of any other toxic components if present. Purification of the enzyme should be facilitated by its high pI which allows use of ion exchange chromatography.

As wilting of infected plants is a major symptom and presumably main cause of yield loss in this disease, the causative factor should be investigated. Measurement of water potential using for example a Scolander pressure bomb (Scolander, 1965) should indicate whether wilting is caused by water stress due to xylem vessel blockage or by membrane damage due to a toxin. In this method, a decreased water potential would indicate water stress. In view of the evidence for production of EPS in xylem vessels and for the extreme sensitivity of vascular flow

to trace levels of large polysaccharides (Van Alfen, 1982) occlusion by Xcm EPS is the likely mechanism.

Like other xanthomonads the mode of pathogenicity of Xcm is still only partly understood but this study has laid a firm foundation for further studies of this important pathogen.

APPENDICES

Appendix I: Media

Minimal medium A

K_2HPO_4	10.2g
KH_2PO_4	4.5g
ammonium sulphate	1.0g
sodium citrate	0.5g
agar	14g
distilled water	1l

Sterile 0.5% glucose and sterile 1mM magnesium sulphate is added after autoclaving the above for 15mins at 1.4 bars and 121°C.

Minimal medium B

Glucose	0.5%w/v
$NH_4H_2PO_4$	0.5g
$MgSO_4\ 7H_2O$	0.2g
K_2HPO_4	0.5g
NaCl	2.0g
$CaCl_2\ 2H_2O$	0.1g
distilled water	800ml
agar	14g

Watanabe medium

L glutamic acid	1g
L methionine	0.5g
$(NH_4)_2HPO_4$	3g
KH_2PO_4	2g
$MgCl_2\ 6H_2O$	1g
$FeSO_4\ 7H_2O$	0.01g
$MnSO_4\ 4H_2O$	0.01g
distilled water	1l

Appendix II: Composition of the cassava tuber

(Leslie, 1987).

Food composition of 1kg of cassava-

Food energy (cal)	1460
Water (g)	625
Carbohydrate (g)	347
Protein (g)	12
Fat (g)	3
Ca (mg)	330
Fe (mg)	7
Vit. A (IU)	trace
Thiamine (mg)	0.6
Riboflavin (mg)	0.3
Niacin (mg)	6
Vit. C (mg)	360

Appendix III: Amino acid analysis

1.Total amino acids

i).Acid hydrolysis - for all amino acids except methionine and cysteine

Reagents HCl 6M

Citrate diluting buffer pH 2.2

Procedure

a).Weigh accurately, approximately 100mg of dried and defatted sample into a 250ml quickfit tube.
b).Add 100ml 6M HCl and a few anti-bumping granules.
c).Connect the flask to the condensers and reflux for 24h using the Gerhardt Kjeldatherm.
d).Wash down the condensor with a little deionised water and allow to cool. Transfer the hydrolsate to a 200ml volumetric flask and make upto the mark. Mix well.
e).Filter through Whatman No. 541 filter paper into a plastic bottle. (hydrolsate can be stored frozen in the bottle).
f).Pipette a 5ml aliquot of the filtrate into a rotary evaporator flask and dry at 40°C. Wash a few times with deionised water until the acid is removed.
g).Pipette analiquot of pH 2.2 diluting buffer to the residue according to the nitrogen content of the dried and defatted sample.

% Nitrogen	Aliquot of pH 2.2 buffer
1-5	5ml
6-10	10ml
11-16	15ml

h).Swirl the flask to dissolve the residue and filter through a 0.46um filter into a small container (this is also stored frozen until use).
i).The solution is then loaded onto the amino acid analyser for separation.

ii).Oxidation- for methionine and cysteine

Reagents 88% formic acid (98% formic acid made upto 100ml with deionised water)
Hydrogen peroxide solution (30%w/v)
Performic acid- 5ml H_2O_2 to 45ml 88% formic acid
Sodium metabisulphite
Conc. HCl
Diluting buffer pH 2.2

Procedure

a).Make up sufficient performic acid for the number of samples to be analysed. Stand at room temperature for 1h and cool to 0°C.
b).Weigh approx. 100mg of dried and defatted sample into a 250ml quickfit tube.
c).Stand the tubes in ice and add 10ml of performic acid. Cover the tubes with parafilm and leave in a fridge at 0-4°C overnight.
d).Add 0.88g of sodium metabisulphite in small amounts and wait until effervescence ceases.
e).Add 20ml of deionised water followed by 30ml conc. HCl. Add a few anti-bumping granules.
f).Connect to a condenser and reflux for 22h.
g).Wash down the condenser with a small amount of deionised water, cool and make upto 100ml.
h).Filter through Whatman No. 541 filter paper into plastic bottles.
i).Pipette a 5ml aliquot and take down to dryness on a rotary evaporator at 40°C. Wash a few times with deionised water until the acid is removed.
j).Add 5ml of pH 2.2 buffer to the residue, swirl to dissolve and filter through a 0.45um filter (store frozen until use).
k).Load onto the amino acid analyser for separation.

2.Extraction of free amino acids (Draper, 1976).

a).Macerate 5g of leaf tissue with 50ml of 80% EtOH.
b).Filter the slurry through paper and extract residue with two 50ml portions of EtOH.
c).Reduce the ethanolic extract to dryness in vacuo at 40°C and shake the residue with a mixture of water (25ml) and light petrolium (b.p. 40-60°C).
d).Discard the upper layer and repeat the procedure twice.
e).Dry the final pigment-free aqueous phase and dissolve the amino acids in 0.1M HCl.

Appendix IV- Free amino acids (mg/g sample on dry matter basis)

Sample	control	water control	necrotic area	adjacent area
Aspartic acid	0.16	0.13	0.075	0.15
Threonine	0.11	0.08	-	0.13
Serine	0.07	0.10	-	0.10
Glutamic acid	0.27	0.13	0.075	0.13
Glycine	0.007	0.013	0.016	0.022
Alanine	0.008	0.083	0.08	0.087
Valine	0.02	0.02	0.09	0.05
Isoleucine	0.007	0.01	0.01	0.02
Leucine	0.01	0.02	0.01	0.03
Tyrosine	0.008	0.01	0.03	0.02
Phenyl alanine	0.01	0.03	0.05	0.08
Histidine	0.04	0.08	0.08	0.08
Arginine	0.06	0.08	0.06	0.09
Proline	-	0.03	0.28	0.07
Lysine	0.007	0.01	0.004	0.01

Appendix V

Other degradative enzymes of Xcm

An early attempt was made to determine potential range of extracellular degradative enzymes of a range of isolates of Xcm.

Glucanase

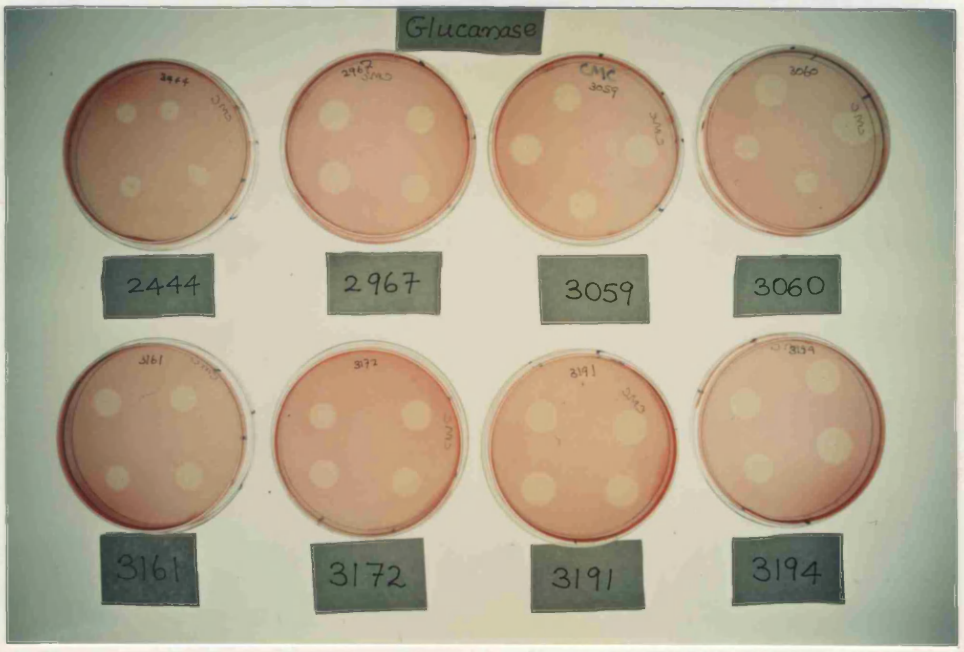
Screening for glucanase activity was done using the method of Wood (1981).

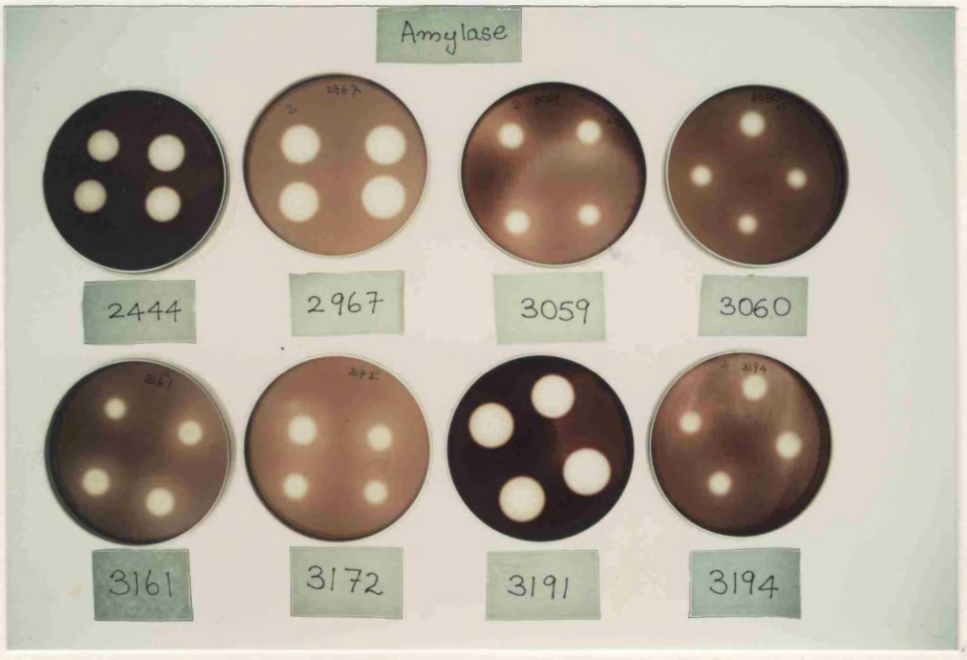
All eight Xcm isolates showed similar glucanase activity.

Amylase -

Amylase production was evaluated by growing the bacterial isolates on NYGA containing 0.1% (w/v) starch (soluble, Analar grade, BDH). After incubation (30°C, 2 days), plates were flooded with 1:100 dilution of 0.08M I_2 in 3.2M KI. Amylase activity was shown by cleared zones around colonies.

All eight Xcm isolates produced amylase but the isolates 3191, 2967and 2444 demonstrated most amylase activity.


Glucanase
2444
2967
3059
3060
3161
3172
3191
3194


Amylase
2444
2967
3059
3060
3161
3172
3191
3194

Appendix VI

Tobacco plant infiltrated with Xcm isolate 2967 and SDW (control). The plant infiltrated with Xcm showed an HR after 48h.


Infiltration
2967
Infiltration
SDW

BIBLIOGRAPHY

Abo-El-Dahab,M.J. (1964). Production of pectic and cellulolytic enzymes by Xanthomonas malvacearum. Phytopathology 54, 597-601.

Akazawa,T. & Wada,K. (1961). Analytical study of Ipomeamarone & chlorogenic acid alterations in sweet potato roots infected by Ceratosystis fimbriata. Plant Physiology 36,139-144.

Allington,W.B., Chamberlain,D.W.(1949). Trends in the population of pathogenic bacteria within leaf tissues of susceptible and immune plant species. Phytopathology 39, 656-660.

Amaral,J.F. (1942). Estudo do organismo causador de bacteriose da mandioca. Arquivos Instituto Biologico, Sao Paulo. 13, 119-126.

Amaral,J.F. (1945). Doencas vasculares das plantas causadas por bacterias. O.Bilogico, Sao Paulo 11, 250-253.

Andro,T., Chambost,J.P., Kotoujansky,A., Cattaneo,J., Bertheau.Y., Barras,F. & Coleno,A. (1984). Mutants of Erwinia chrysanthemi defective in secretion of pectinase and cellulase. Journal of Bacteriology 160, 1199-1203.

Angeles-Ramos,R. Vidaver,A.K. & Flynn,P. (1991). Characterization of epiphytic Xanthomonas campestris pv phaseoli and pectolytic xanthomonads recovered from symptomless weeds in the Dominican Republic. Phytopathology 81, 677-681.

Arnold,M.H. & Brown,S.J. (1968). Variation in the host-parasite relationship of a crop disease. Journal of Agricultural Science 71, 19-36.

Atkinson,M.M., Huang,J. & Knopp,J.A. (1985). Hypersensitivity of suspension-cultured tobacco cells to pathogenic bacteria. Phytopathology 75, 1270-1274.

Ayres,A.R., Ayers,S.B. & Goodman,R.N. (1979). Extracellular polysaccharide of Erwinia amylovora: a correlation with virulence. Applied and Environmental Microbilogy 38, 659-666.

Bailey,J.A.(1970). Pisatin production by tissue cultures of Pisum sativum L. Journal of General Microbiology 6, 409-415.

Ballio,A., Chain,E.B., DeLeo,P., Erlanger,B.F., Kauri,M. & Tonolo,A. (1964). Fusicoccin: a new wilting toxin produced by Fusicoccum amygdali. Nature 203, 297.

Barrëre,G.C., Barber,C.E. & Daniels,M.J. (1986). Molecular cloning of genes involved in the production of the extracellular polysaccharide xanthan by Xanthomonas campestris pv campestris. International Journal of Biology of Macromolecules 8, 372-374.

Basham,H.G. & Bateman, D.F. (1975a). Relationship of cell death in plant tissue treated with a homogeneous endo pectate lyase to cell wall degradation. Physiological Plant Pathology 5, 249-262.

Basham,H.G. & Bateman,D.F. (1975b). Killing of plant cells by pectic enzymes; the lack of direct injurious interaction between pectic enzymes or their soluble reaction products and plant cells. Phytopathology 65, 141-153.

Bashan,Y., Okon,Y. & Henis,Y. (1986). A possible role for proteases and deaminases in the development of the bacterial speck disease in tomato caused by Pseudomonas syringae pv tomato. Academic Press, London.

Bateman,D.F. (1963). Pectolytic activities of culture filtrates of Rhizoctonia solani and extracts of Rhizoctonia infected tissues of bean. Phytopathology 53, 197-204.

Bateman,D.F. (1968). The enzymatic maceration of plant tissue. Netherlands Journal of Plant Pathology 74, 67-80.

Bateman,D.F. (1976). Plant cell wall hydrolysis by plant pathogens. In: Biochemical aspects of plant-parasite relationships. Ed: Friend,J. & Threlfall,D.R. 79-103 pp. Academic Press, London.

Barnett,N.M. & Naylor,A.W. (1966). Amino acid and protein metabolism in Bermudagrass during water stress. Plant Physiology 41, 1222-1230.

Bateman,D.F. & Basham,H.G. (1976). Degradation of plant cell walls and membranes by microbial enzymes. In: Encyclopedia of Plant Pathology. Ed:R.Heitfuss and P.H.Williams. 316-355pp. Springer Verlag, New York.

Beaulieu,C., Minsavage,G.V., Canteros,B.I. & Stall,R.E. (1991). Biochemical and genetic analysis of a pectate lyase gene from Xanthomonas campestris pv vesicatoria. Molecular Plant-Microbe Interactions 4(5), 446-451.

Beckman,C.H. (1969). The mechanics of gel formation by swelling of simulated plant cell wall membranes and perforation plates of bannana root vessels. Phytopathology 59, 837-843.

Beckman,C.H. (1971). The plasticizing of plant cell walls and tylose formation- a model. Physiological Plant Pathology 1, 1-10.

Beckman,C.H., Elgersma,,D.M. & Machardy,W.E. (1972). The localisation of fusarial infections in the vascular tissue of single-dominant gene resistant tomatoes. Phytopathology 62, 1256-1260.

Behnke,M. (1979). Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theoritical and Applied Genetics 55, 69-71.

Beltra,R. (1961). Efecto morfoginetico observado en los extractos hormonales de los tumores del olivo. Microbiologia Espanola 14, 177-187.

Bender,C.L., Stone,H.E., Simms,J.J. & Cooksey,D.A. (1987). Reduced pathogen fitness of Pseudomonas syringae pv tomato Tn5 mutants defective in coronatine production. Physiological and Molecular Plant Pathology 30, 273-283.

Bennett,R.A. (1980). Evidence for two virulence determinants in the fire blight pathogen Erwinia amylovora. Journal of General Microbiology 116, 351-356.

Bennett,R.A. & Billing,E. (1980). Origin of the polysaccharide ooze from plants infected with Erwinia amylovora. Journal of General Microbiology 116, 341-349.

Bertheau,Y., Madgidi-Hervan,E., Kotoujansky,A., Nguyen,C., Andro,T. & Coleno,A. (1984). Detection of depolymerase isoenzymes after electrophoresis or electrofocusing or in titration curves. Annals of Biochemistry 139, 383-389.

Biggs,B.J., Smith,M.K. & Scott,K.J. (1986). The use of embryo culture for the recovery of plants from cassava (Manihot esculenta) seeds. Plant Cell, Tissue and Organ Culture 6, 229-234.

Bishop,C.D. & Cooper,R.M. (1983). An ultrastructural study of vascular colonisation in three vascular wilt diseases. I. Colonization of susceptible cultivars. Physiological Plant Pathology 23, 323-343.

Billing,E. (1984). Studies on avirulent strains of Erwinia amylovora. Acta Horticulturae 151, 249-253.

Binarova,P., Nedelnik,J. Fellner,M. & Nedbalkova,B. (1990). Selection for resistance to filtrates of Fusarium spp. in embryogenic cell suspension cultures of Medicago sativa L. Plant Cell, Tissue and Organ Culture 22, 191-196.

Bhushan,A. & Prasad,M. (1987). Pathochemical studies of cowpea, infected with Xanthomonas campestris pv vignicola and the role of laef amino acids in evoking susceptible and resistant responses. Zentralbl. Mikrobiology 142, 621-629.

Boccara,M., Diolez,A., Rouve,M. & Kotoujansky,A. (1988). The role of individual pectate lyases of Erwinia chrysanthemi strain 3937 in pathogenicity on saintpaulia plants. Physiological and Molecular Plant Pathology 33, 95-104.

Boher,B. & Daniel,J.F. (1985). Identification of some sites of expression of tolerance to Xanthomonas campestris pv manihotis (Arthaud-Berthet) Starr in some cassava (Manihot esculenta Crantz) cultivars. Agronomie 5(8), 677-683.

Bondar,G. (1912). Una nova molestia bacteriana das hastes da mandioca. Chacaras e Quinto Sao Paulo 5, 15-18.

Bouriquet,G. (1949). Pathologie du manioc dars les territories Francais d'Outre-mer. In: Congres du manioc Merseille, Institut Colonial 73-75 pp.

Bradshaw-Rouse,J.J., Whatley,M.A., Coplin,D.L., Woods,A., Sequeira,L. & Kelman,A. (1981). Agglutination of strains of Erwinia stewartii with a corn agglutinin: Correlation with extracellular polysaccharide production and pathogenicity. Applied and Environmental Microbiology 42, 344-350.

Braun,E.J. (1990). Colonisation of resistant and susceptible maize plants by Erwinia stewartii strains differing in exopolysaccharide production. Physiological and Molecular Plant Pathology 36, 363-379.

Braun,E.J. (1982). Ultrastructural investigation of resistant and susceptible maize inbreds infected with Erwinia stewartii. Phytopathology 72, 159-166.

Bretschneider,K.F., Gonella,M.P. & Robeson,D.J. (1990). A comparative light and electron microscopical study of compatible and incompatible interactions between Xanthomonas campestris pv campestris and cabbage (Brassica oleracea). Physiological and Molecular Plant Pathology 34, 285-297.

Brettel,R. Ingram,D.S. & Thomas,E. (1980). Selection of maize tissue cultures resistant to Drechslera (Helminthosporium) maydis T-toxin. In: Tissue Culture Methods for Plant Pathologists. Ed: Ingram,D.S. & Helgeson,J.P. 233-237 pp. Blackwell, Oxford.

Brian,P.W., Elson,G.W., Hemming,H.G. & Wright,J.M. (1952). The phytotoxic properties of alternaric acid in relation to etiology of plant diseases caused by Alternaria solani Ell & Mart.) Jones & Grout. Annals of Applied Biology 39, 308-321.

Briggs,D.E. (1966). Gibberellin-like activity of helminthosporol and helminthosporic acid. Nature 210, 418-419.

Brisset,M.N. & Paulin,J.P. (1991). Relationships between electrolyte leakage from Pyrus communis and virulence of Erwinia amylovora Physilogical and Molecular Plant Pathology 39, 443-453.

Brown,I.R. & Mansfield,J.W. (1988). An ultrastructural study, including cytochemistry and quantitative analysises, of the interactions between pseudomonads and leaves of Phaseolus vulgaris L. Physilogical and Molecular Plant Pathology 33, 351-376.

Buchanan,G.E. & Starr,M.P. (1980). Phytotoxic material from associations between Erwinia amylovora and pear tissue culture: Possible role in necrotic symptomology of fire blight disease. Current Microbiology 4, 63-68.

Burkholder,W.H. (1942). Three bacterial plant pathogens: Phytomonas caryophilli sp. n., Phytomonas alliicola sp.n., and Phytomonas manihotis (Arthaud-Berthet et Bondar) Viegas. Phytopathology 32, 141-149.

Burkholder,W.H. & Starr,M.P. (1948). The genetic and specific charachters of phytopathogenic species of Peudomonas and Xanthomonas. Phytopathology 38, 494-502.

Butcher,D.N., Firmin,J.L. & Searle,L.M. (1980). The role of tissue culture in the study of crown-gall tumerigenesis. In: Tissue Culture Methods for Plant Pathologists. Ed: Ingram,D.S. & Helgeson,J.P. 203-208 pp. Blackwell, Oxford.

Carneiro,J.G. (1940). Doenca bacteriana da mandioca. Notas e Informacoes. Portaria do Ministerio Agricultura, Sao Paulo 573, 447.

Castano,J.J. (1969). Monicha foliar de Cercospora caribaea en yuca (Manihot utilissima Pohl) en la region de Barbosa (Anbioquia). Agricultura Tropical Bogota, 25, 327-329.

Chagas,C.M., Rossetti,V.& Beretta,M.J.G. (1992). Electron microscopy studies of a xylem-limited bacterium in sweet orange affected with Citrus variegated chlorosis disease in Brazil. Journal of Phytopathology 134, 306-312.

Chant,S.R. (1959). A note on the inactivation of mosaic virus in cassava (Manihot utilissima Pohl.) by heat treatment. Empire Journal of Experimental Agriculture 27, 55-58.

Chatterjee,A.K. Ross,M. & Sanderson,K.E. (1976). Leakage of periplasmic enzymes from lipopolysaccharide defective mutants of Salmonella typhimurium. Canadian Journal of Microbiology 22, 1549.

Chatterjee,A.K. & Vidaver,A.K. (1986). Genetics of pathogenecity factors: application to phytopathogenic bacteria. In: Advances in Plant Pathology Vol.4. 1-224 pp. Academic Press, New York.

Chen,H. Gray,J.X., Nayudu,M., Djordjevic,M.A., Batley,M., Redmond,J.W. & Rolfe,B.G. (1988). Five genetic loci involved in the synthesis of acidic exopolysaccharides are closely linked in the genome of Rhizobium sp. strain NGR 234. Molecular and General Genetics 211, 310-316.

Chen,W.H., Lieu,M.Ch., & Chao,Ch.Y. (1979). The growth of sugarcane downy mildew fungus in tissue culture. Canadian Journal of Botany 57, 528-533.

Chërif,M., Benhamou,N. & Belanger,R.R. (1991). Ultrastructural and cytochemical studies of fungal development and host reactions in cucumber plants infected by Pythium ultimum. Physiological and Molecular Plant Pathology 39, 353-375.

Chiang,M.S. & Nipp,W.K. (1973). Free amino acid content in leaf and root tissues of club-root resistant and club-root susceptible cabbages. Euphytica 22, 293-298.

CIAT (1972). CIAT Annual Report 1972, CIAT, Cali, Colombia.192pp.

CIAT (1975). CIAT Annual Report 1975, CIAT, Cali, Colombia. B-15-B-18pp.

CIAT (1976). CIAT Annual Report 1976, CIAT, Cali, Colombia. 120pp.

Cock,J.H. (1985). Cassava-New Potential for a neglected crop. 191pp. Westview Press, London.

Cohen,B.L. (1981). Regulation of protease production in Aspergillus. Transactions of the British Mycological Society 76, 447-450.

Cohen,B.L. & Drucker,M. (1977). Regulation of exocellular protease in Neurospora crassa: Induction and repression under conditions of nitrogen starvation. Archives of Biochemistry and Biophysics 182, 601-613.

Collmer,A., Berman,P. & Mount,M.S. (1982). Pectate lyase regulation and bacterial soft rot pathogenesis. In: Phytopathogenic Prokaryotes, Vol. I. Eds: Mount,M.S. & Lacy,G.H. 395-442pp. Academic Press, New york.

Collmer,A., Schodel,C., Roeder,D.L., Ried,J.L. & Rissler,J.F. (1985). Molecular cloning in Escherichia coli of Erwinia chrysanthemi genes encoding mutiple forms of pectate lyase. Journal of Bacteriology 161(3), 913-920.

Comstock,J.C. & Scheffer,R.P. (1972). Production and relative host specificity of a toxin from Helminthosporium maydis race T. Plant Disease Reporter 56, 247-51.

Cook,A.A. & Stall,R.E. (1968). Effect of Xanthomonas vesicatoria on loss of electrolytes from leaves of Capsicum annum. Phytopathology 58, 617-619.

Cooper,R.M. (1974). Cell wall degrading enzymes of vascular wilt fungi. Ph.D. thesis, University of London.

Cooper,R.M. (1977). Regulation of synthesis of cell wall-degrading enzymes of plant pathogens. In: Cell wall biochemistry related to specificity in host-plant pathogen interactions. Eds: Solheim,B. & Raa,J. 163-211pp. Universitatsforlaget, Oslo.

Cooper,R.M. (1983). the mechanisms and significance of enzymic degredation of host cell walls. In: Biochemical Plant Pathology. Eds: Callow,J.A. 101-137pp. Wiley, New York.

Cooper, R.M. (1984). The role of cell wall-degrading enzymes in infection and damage. In: Plant Diseases: Infection, Damage and Loss Eds: R.K.S.Wood and G.J.Jellis. 13-28pp. Blackwell, Oxford.

Cooper,R.M. (1987). The use of mutants in exploring depolymerases as determinants of pathogenicity. In: Genetics and Plant Pathogenesis. Eds: Day,P.R. & Jellis,G.J. 261-281pp. Blackwell, Oxford.

Cooper,R.M. (in press). Toxins in plant disease. In: Physiological Plant Pathology Ed: R.K.S. Wood Blackwell, Oxford.

Cooper,R.M. & Wood,R.K.S. (1975). Regulation of synthesis of cell wall degrading enzymes by Verticillium albo-atrum and Fusarium oxysporum f.sp. lycopersia. Physiological Plant Pathology 5, 135-156.

Cooper,R.M. & Wood,R.K.S. (1980). Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endopectin lyase in Verticillium wilt of tomato. Physiological Plant Pathology 16, 285-300.

Coplin,D.L. & Cook,D. (1990). Molecular genetics of extracellular polysaccharide biosysnthesis in vascular phytopathogenic bacteria. Molecular Plant-Microbe Interactions 3(5), 271-279.

Coplin,D.L. & Majerczak,D.R. (1990). Extracellular polysaccharide genes in Erwinia stewartii: directed mutagenesis and complementation analysis. Molecular Plant-Microbe Interactions 3, 286-292.

Costa,A.S., Kitajima,E.W., Pereira,A.S., Silva,J.R. & Carvalho Diaz,C.A. (1970). Molestias de virus de micoplasma da mandioca no estado de Sao Paulo. Boletin Secretaria de Agricultura Industria and Comercio, Sao Paulo. 18pp.

Costerton,J.W., Cheng,K.J., Geesey,G.G. Ladd,T.I., Nickel,J.C., Dasgupta,M. & Marrie,T.J. (1987). Bacterial biofilms in nature and disease. Annual Review of Microbiology 41, 435-464.

Costerton,J.W., Ingram,J.M. & Cheng,K.J. (1974). Structure and function of the cell envelope of gram-negative bacteria. Bacteriological Reviews 38, 87-110.

Crosse,J.E. (1966). Epidemiological relations of the pseudomonad pathogens of deciduous fruit trees. Annual Review of Phytopathology 4, 291-306.

Daly,J.M. (1987). Toxins as determinants in plant disease. In: Molecular Determinants of Plant disease Ed: S.Nishimura et al., 119-126pp. Springer Verlag, Berlin.

Daniels,M.J., Barber,C., Turner,P., Cleary,W. & Sawzcyc,M. (1984). Isolation of mutants of Xanthomonas campestris pv campestris showing altered pathogeneicity. Journal of General Microbiology 130, 2447-2455.

Daniels,M.J., Osbourn,A.E. & Tang,J.L. (1989). Regulation in Xanthomonas-plant interactions. NATO ASI Series Vol. H 36. Eds: Luglenberg,B.J.J. 189-196pp. Springer-Verlag, Berlin.

Daub,M.E. (1986). Tissue culture and the selection of resistance in pathogens. Annual Review of Phytopathology 24, 159-186.

Davies,R. (1963). Microbial extracellular enzymes, their uses and some factors affecting their formation. In: Biochemistry of industrial microorganisms. Eds: Rainbow,C. & Rose,A.H. 68-150pp. Academic Press, New York.

Day,P.R. (1980). Tissue culture methods in plant breeding. In: Tissue Culture Methods for Plant Pathologists Eds: Ingram,D.S. & Helgeson,J.P. 223-231pp. Blackwell, Oxford.

Dekhuijzen,H.M. (1975). The enzymatic isolation of secondary vegetative plasmodia of Plasmodiophora brassicae from callus tissue of Brassica campestris Physiological Plant Pathology 6, 187-192.

Dekker,R.F.H., & Candy,G.P. (1979). the β-mannases elaborated by the phytopathogen Xanthomonas campestris. Archives of Micrbiology 122, 297-299.

DeVay,J.E., Lukezic,F.L., Sinden,S.L., English,H. & Coplin,D.L. (1968). A biocide produced by pathogenic isolates of Pseudomonas syringae and its possible role in the bacterial canker disease of peach trees. Phytopathology 58, 95-101.

Diener,T.O. (1960). Free amino acids and amides in healthy and virus-infected cherry and peach leaves. Phytopathology 50, 141-145.

Dimmond,A. & Wggoner,P.C. (1953). On the nature and role of vivotoxins in plant diseases. Phytopathology 43, 229-235.

Dimond,A.E. (1955). Pathogenesis in the wilt diseases. Annual Review of Plant Physiolology 6, 329-50.

Dimond,A.E. (1972). The origin and symptoms of vascular wilt. In: Phytotoxins in Plant Diseases. Eds: Wood,R.K.S. Ballio,A. & Graniti,A. 289-309pp. Academic Press, London.

Dixon,R.A. (1980). Plant tissue culture methods in the study of phytoalexin induction. In: Tissue Culture Methods for Plant Pathologists Eds: Ingram,D.S. & Helgeson,J.P. 223- 231pp. Blackwell, Oxford.

Dixon,R.A. & Brendall,D.S. (1978). Changes in phenolic compounds associated with phaseollin production in cell suspension cultures of Phaseolus vulgaris. Physiological Plant Pathology 13, 283-294.

Dixon,R.A. & Fuller,K.W. (1976). Effects of synthetic auxin levels on phaseollin production and phenylalanine ammonia-lyase (PAL) activity in tissue cultures of Phaseolus vulgaris L. Physiological Plant Pathology 9, 299-312.

Dixon,R.A. & Fuller,K.W. (1978). Effects of growth susbstances on non-induced and Botrytis cinerea culture filtrate induced phaseollin production in Phaseolus vulgaris cell suspension cultures. Physiological Plant Pathology 12, 279-288.

Dixon,G.R. & Pegg,G.F. (1969). Hyphal lysis and tylose formation in tomato cultivars infected by Verticillium albo-atrum. Transactions of the British Mycological Society 53, 109-118.

Djordjevic,S.P., Chen,H., Batley,M., Redmond,J.W. & Rolfe,B.G. (1987). Nitrogen fixation ability of exopolysaccharide synthesis mutants of Rhizobium sp. strain NGR 234 and Rhizobium trifolii is restored by the addition of homologous exopolysaccharides. Journal of Bacteriology 169, 53-60.

Dolph,P.J., Majerczak,D.R. & Coplin,D.L. (1988). Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in Erwinina stewartii. Journal of Bacteriology 162, 504-509.

Dow,J.M., Scofield,G. Trafford,K. Turner,P.C. & Daniels,M.J. (1987). A gene cluster in Xanthomonas campestris pv campestris required for pathogenicity controls the excretion of polygalacturonate lyase and other enzymes. Physiological and Molecular Plant Pathology 31, 261-271.

Dow,J.M., Milligan,D.E., Jamieson,L. Barber,C.E, & Daniels,M.J. (1989). Molecular cloning of a polygalacturonate lyase gene from Xanthomonas campestris pv campestris and role of gene product in pathogenicity. Physiological and Molecular Plant Pathology 35, 113-120.

Dow,J.M., Clarke,B.R., Milligan, D.E., Tang,J.L. & Daniels,M.J. (1990). Extracellular proteases from Xanthomonas campestris pv campestris the black rot pathogen. Applied and Environmental Microbiology 56(10), 2994-2998.

Drews,G., Ziser,K., Schrock-Vietor,U. & Golecki,J.R. (1988). Cellular responses of soybean to virulent and avirulent strains of Pseudomonas syringae pv glycinea. European Journal of Cell Biology 46, 369-375.

Drummond,O.A. & Hipolito,O. (1941). Notas sobre bacteriose da mandioca. Boletin Esccola Superior de Agricultura, Minas Gerais 4, 86-124.

Durbin,R.D. (1983). The biochemistry of fungal and bacterial toxins and their modes of action. In: Biochemical Plant Pathology Ed: J.A.Callow, 137-162pp. John Wiley, Chichester.

Durbin,R.D. & Mathews,D.E. (1987). Bacterial toxins: The mechanism of chlorosis induction by tagetitoxin Current Topics in Plant Biochemistry and Physiology 6, 46-54.

Duron,M., Paulin,J.P. & Brisset,M.N. (1987). Use of in vitro propagated plant material for rating fire blight susceptibility. Acta Horticulturae 217, 317-324.

Dye,D.W. (1960). Pectolytic activity in Xanthomonas. New Zeland Journal of Science 3, 61-69.

Ebel,J., Ayers,A.A. & Albersheim,P. (1976). Host pathogen interactions XII. Response of suspension cultured soybean cells to the elicitor isolated from Phytophthora megasperma var. sojae, a fungal pathogen of soybean. Plant Physiology 57, 775-779.

Eden Green,S.J. (1972).Studies in fire blight disease in pear, apple ang hawthorn. Ph.D. thesis, University of London.

El-Banoby,F.E. & Rudolph,K. (1979). Induction of water soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads. Physiological Plant Pathology 15, 341-349.

El-Banoby,F.E. & Rudolph,K. (1980). Agglutination of Pseudomonas phaseolicola by bean leaf extracts (Phaseolus vulgaris). Phytopathologische Zeitschrift 98, 91-95.

El-Banoby,F.E. & Rudolph,K. (1981). Correlation between degree of agglutination of Pseudomonas phaseolicola cells by raw lectin preparations from 15 bushbean cultivars and degree of resistance to bacterial infection. Phytopathology 71, 872 (abstr.).

El-Banoby,F.E. & Rudolph,K. & Hottermann,A. (1980). Biological and physical properties of an extracellular polysaccharide from Pseudomonas phaseolicola. Physiological Plant Pathology 17, 291-301.

El-Banoby,F.E. & Rudolph,K., Mendgen,K. (1981). The fate of extracellular polysaccharides from Pseudomonas phaseolicola in leaves and leaf extracts from halo-blight susceptible and resistant bean plants (Phaseolus vulgaris L.). Physiological Plant Pathology 18, 91-98.

Elgersma,D.M. (1973). Tylose formation in elms after inoculation with Ceratocystis ulmi. Netherlands Journal of Plant Pathology 79, 218-220.

Ellingboe,A.H. (1976). Genetics of host-parasite interaction. In: Encyclopedia of Plant Physiology Eds: Heitefuss,R. & Williams,P.H. 761-778pp. Springer-Verlag, Stuttgart.

Ercolani,G.L. & Crosse,J.E. (1966). Growth of Pseudomonas phaseolicola and related plant pathogens in vivo. Journal of General Microbiology 45, 429-439.

Ewbank,E. & Maraite,H. (1990). Conversion of methionine to phytotoxic 3 methylthiopropionic acid by Xanthomonas campestris pv manihotis. Journal of General Microbiology 136, 1185-89.

Fagg,J., Wood-Tor,A. & Mansfield,J. (1991). Comparative study of interactions between Bremia lactucae and lettuce cells in suspension culture and in cotyledons. Physiological and Molecular Plant Pathology 38, 105-116.

Favali,M., Conti,G.G. & Bassi,M. (1978). Modifications of the vascular bundle ultrastructure in the 'resistant zone' around necrotic lesions induced by tobacco mosaic virus. Physiological Plant Pathology 13, 247-251.

Fawole,M.O. & Ikotun,T. (1978). The production of polygalacturonase by Xanthomonas manihotis. Acta Phytopathologica 13, 383-391.

Feldman,A.W. & Hanks,R.W. (1964). Quantitative changes in free and protein amino acids in roots of healthy, Rodopholus similis infected and 'recovered' grapefruit seedlings. Phytopathology 54, 1210-1215.

Ferris,G.G. & Beveridge,T.J. (1985). Functions of bacterial cell surface structures. BioScience 35, 172-177.

Fett,W.F., Osman,S.F. & Dunn,M.F. (1989). Characterization of exopolysaccharides produced by plant-associated fluorescent pseudomonads. Applied and Environmental Microbiology 55, 579-583.

Fett,W.F. & Zacharius,R.M. (1982). Bacterially-induced glyceollin production in soybean cell suspension cultures. Plant Science Letters 24, 303-309.

Fett,W.F. & Zacharius,R.M. (1983). Bacterial growth and phytoalexin elicitation in soybean cell suspension

cultures inoculated with Pseudomonas syringae pathovars. Physiological Plant Pathology 22, 151-172.

Food and Agriculture Organization (FAO) (1991). Food balance sheets 1984-90. Rome.

Friedman,B.A. (1962). Physiological differences between a virulent and a weakly virulent radiation-induced strain of Erwinia carotovora. Phytopathology 52, 328-332.

Friedman,B.A. & Jaffee,M.J. (1960). Effect of soft rot bacteria and pectolytic enzymes on electricla conductance of witloof chicory tissue. Phytopathology 50, 272-274.

Fry,S.M. & Milholland,R.D. (1990). Response of resistant, tolerant and susceptible grapevine tissues to invasion by the Pierce's disease bacterium, Xylella fastidiosa. Phytopathology 80, 66-69.

Gainor,C. & Crisley,F.D. (1961). Proteolytic activity of crude stem extracts from normal and tumor tissues of plants. Nature 190, 1031-1032.

Gardner,J.M. & Kado,C.I. (1976). Polygalacturonic acid trans-eliminase in the osmotic shock fluid of Erwinia rubrifaciens. Characterisation of the purified enzyme and its effect on plant cells. Journal of Bacteriology 127, 451-460.

Gaumann,E. (1957). Fusaric acid as a wilt toxin. Phytopathology 47, 342-357.

George,H.L., Mount,M.S. & Berman,P.M. (1991). Cellular localization and characterization of pectic enzymes of Erwinia carotovora subsp. atroseptica. Phytopathology 81, 134-139.

Gilchrist,D.G. (1983). Molecular modes of action. In: Toxins and Plant Pathogenesis Eds: Daly,J.M. & Deverall,B.J. 81-136pp.

Ginzberg,B.Z. (1961). Evidence for a protein gel structure cross-linked by metal cations in the intercellular cement of plant tissues. Journal of Experimental Botany 12, 85-107.

Giovanelli,J., Owens,L.D. & Mudd,S.H. (1972). β cystathionase in vivo inactivation by rhizobitoxin and role of the enzyme in methionine biosynthesis in corn seedlings. Plant Physiology 51, 492-503.

Gnanamanickam,S.S., Starratt,A.N. & Ward,E.W.B. (1982). Coronatine production in vitro and in vivo and its relation to symptom development in bacterial blight of soybean. Canadian Journal of Botany 60, 645-650.

Gonclaves,R.D. (1939). A bacteriose da mandioca no vale do Pariba. Notas e Informacoes. O Biologico, Sao Paulo 5, 117-118.

Gonclaves,R.D. (1948). Bacteriose de mandioca. Notas e Informacoes. O Biologico, Sao Paulo 14, 145-146.

Goodman,R.N. (1982). The infection process. In: Phytopathogenic Prokaryotes, Vol I Eds: Mount,M.S. & Lacy,G.H. 31-62pp. Academic Press, London.

Goodman,R.N. & White,J.A. (1981). Xylem parenchyma plasmolysis and and vessel wall disorientation are early signs of pathogenesis caused by Erwinia amylovora. Phytopathology 71, 844-852.

Gross,D.G. & Cody,Y.S. (1985). Mechanism of plant pathogenesis by Pseudomonas species. Canadian Journal of Microbiology 31, 403-410.

Gross,M. & Rudolph,K. (1987). Studies on the extracelluar polysaccharide produced in vitro by Pseudomonas phaseolicola Journal of Phytopathology 119, 289-297.

Grout,B.W.W. & Weatherhead,M.A. (1980). A strategy for the production of disease resistant mutants. In: Tissue Culture Methods for Plant Pathologists Eds: Ingram,D.S. & Helgeson,J.P. 249- 254pp. Blackwell, Oxford.

Gustine,D.L., Sherwood,R.T. & Vance,C.P. (1978). Regulation of phytoalexin synthesis in jackbean callus cultures. Stimulation of phenylalanine ammonia-lyase and O-methyltransferase. Plant Physiology 61, 226-230.

Hagihara,B. (1960). Bacterial and mold proteases. In: The Enzymes Vol.IV. Eds: Boyer,P.D., Cardy,H. & Myrback,K. 193-213pp. Academic Press, New York.

Hall,J.A. & Wood,R.K.S. (1970). Plant cells killed by soft rot parasites. Nature 227, 1266-1277.

Hammerschlag,F.A. (1984). In vitro approaches to disease resistance. In: Applications of Genetic Engineering to Crop Improvement Eds: Collins,G.B. & Petolino,J.B. 453-490pp. Martinus Nijhoff/Junk, Netherlands.

Hammerschlag,F.A. (1988). Selection of peach cells for insensitivity to culture filtrates of Xanthomonas campestris pv pruni and regeneration of resistant plants. Theoritical and Applied Genetics 76, 865-869.

Hanks,R.W. & Feldman,A.W. (1966). Quantitative changes in free and protein amino acids in leaves of healthy, Rodopholus similis infected and 'recovered' grapefruit seedlings. Phytopathology 56, 261-264.

Hansford,C.G. (1938). Annual report of the Plant Pathologist, Report of Department of Agriculture, 1936-37 (11), 43-49.

Harding,N.E., Cleary,J.M., Cabanas,D.K., Rosen,R.G. & Kang,K.S. (1987). Genetic and physical analyses of a cluster of genes essential for xanthan gum biosynthesis in Xanthomonas campestris. Journal of Bacteriology 169, 2854-2861.

Harper,S., Zewdie,N., Brown,I.R. & Mansfield,J.W. (1987). Histological, physiological and genetical studies of the responses of leaves and pods of Phaseolus vulgaris to three races of Pseudomonas syringae pv phaseolicola Pseudomonas syringae pv coronafaciens. Physilogical and Molecular Plant Pathology 31, 153-172.

Hashimoto,T. & Tamura,S. (1967). Physiological activities of helminthosporal and helminthosporic acid. II.Effects of excised plant parts. Plant Cell Physiology 8, 35-45.

Hayward,A.C., Teakle,D.S., Saxena,K.B., Wallis,E.S. & Moffet,M.L. (1983). Report of halo blight on pigeonpea from Australia. International Pigeon Pea Newsletter 54-55.

Helgeson,J.P., Kemp,J.D., Haberlach,G.T. & Maxwell,B.P. (1972). A tissue culture system for studing disease resistance: the black shank disease in tobacco callus cultures. Phytopathology 62, 1439-1443.

Helgeson,J.P., Haberlach,G.T. & Upper,C.D. (1976). A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. Phytopathology 66, 91-96.

Helgeson,J.P., Budde,A.D. Haberlach,G.T. (1978). Capsidiol. A phytoalexin produced by tobacco callus tissues. Plant Physiology 61, (Supplement) 53pp.

Helgeson,J.P. (1983). Studies of host-pathogen interactions in vitro. In: Use of Tissue Culture and Protoplasts in Plant Pathology Eds: Helgeson,J.P. & Deverall,J.P. 9-38pp. Academic Press, Sydney.

Heppel,L.A. (1976). Selective release of enzymes from bacteria. Science 156, 1451-1455.

Hignett,R.C. & Quirk,A.V. (1979). Properties of phytotoxic components of plant pathogenic pseudomonads. Journal of General Microbiology 110, 77-81.

Hignett,R.C., Roberts,A.L., Ray,T.C. & Smith,A.R.W. (1983). Biology and biochemistry of Erwinia amylovora. Report of East Malling Research Station for 1982. 79-80pp.

Hilsop,E.C., Keon,J.P.R. & Fielding,A.H. (1979). Effects of pectin lyase from Monilinia fructigena on viability, ultrastructure and localization of acid phosphatase of cultured apple cells. Physiological Plant Pathology 14, 371-381.

Hodgson,R., Peterson,W.H. & Ricker,A.J. (1949). The toxicity of polysaccharides and other large molecules to tomato cuttings. Phytopathology 39, 47-61.

Holliday,M.J. & Klarman,A. (1979). Expression of disease reaction types in soybean callus from resistant and susceptible plants. Phytopathology 69, 576-578.

Hopper,D.G., Venere,R.J. Brinkerhoff,L.A. & Gholson,R.K. (1975). Necrosis induction in cotton. Phytopathology 65, 206-13.

Horton,J.G. & Keen,N.T. (1966). Regulation of induced cellulase synthesis in Pyrenochaeta terrestris by utilisable carbon compounds. Canadian Journal of Microbiology 12, 209-220.

Hotte,B., Ruth-Arnold,I., Puhler,A. & Simon,R. (1990). Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production in Xanthomonas campestris pv campestris. Journal of Bacteriology 172, 2804-2807.

Hsu,S. & Goodman,R.N. (1978). Production of a host specific, wilt inducing toxin in apple cell suspension cultures inoculated with Erwinia amylovora. Phytopathology 68, 351-354.

Huang,J.S. (1980). Galactosyl transferase activity in Erwinia stewratii and its role in biosynthesis of extracellular polysaccharides. Physiological Plant Pathology 17, 73-80.

Huang,P.Y., Huang,J.S. & Goodman,R.N. (1975). Resistance mechanisms to an avirulent strain of Erwinia amylovora. Physiological Plant Pathology 6, 283-287.

Huang,Y., Helgeson,J.P. & Sequeira,L. (1989). Isolation and purification of a factor from Pseudomonas solanacearum that induces a hypersensitive-like response in potato cells. Molecular Plant-Microbe Interactions 2(3), 132-138.

Husain,A. & Kelman,A. (1958). Relation of slime production to mechanism of wilting and pathogenicity of Pseudomonas solanacearum. Phytopathology 48, 155-65.

Ikotun,T. (1975). Cassava bacterial blight disease caused by Xanthomonas manihotis (Arthaud Berthet y Bondar) Starr (CBB). Ph.D. thesis, University of London.

Ikotun,T. (1984a). Inducible production of pectin-methylesterase by Xanthomonas campestris pv manihotis. Zeitschrift fur allgemeine Mikrobiologie 24(3), 139-142.

Ikotun,T. (1978). Effect of Xanthomonas manihotis on cassava tissues. In: Workshop on cassava bacterila blight in Africa, past, present and future. Eds: Terry,E.R., Persley,G.J. & Cook,S.C.A. Centre for Overseas Pest Research, London.

Ikotun,T. (1984b). Pectolytic enzymes produced by Xanthomonas campestris pv cassavae and their involvement in pathogenesis. Zeitschrift fur allgemeine Mikrobiologie 24(6), 263-268.

Ikotun,T. (1987). Leakage of electrolytes from cassava tissue caused by an application of culture filtrate of Xanthomonas campestris pv manihotis. Die Nahrung 31, 305-309.

Ingram,D.S. & Robertson,N.F. (1965). Interaction between Phytophthora infestans and tissue cultures of Solanum tuberosum. Journal of General Microbiology 40, 431-437.

Ingram,D.S. (1969). The susceptibility of Brassica callus to infection by Perenospora parasitica. Journal of General Microbiology 58, 391-401.

Ingram,D.S. & Joachim,I. (1971). The growth of Perenospora farinosa f. sp. betae and sugar beet callus tissues in dual culture. Journal of General Microbiology 69, 211-220.

Ingram,D.S. (1976). Growth of biotrophic parasites in tissue culture. In: Physiological Plant Pathology. Eds: Heitfuss,R. & Williams,P.H. 890pp. Springer-Verlag, Berlin.

Ingram,D.S. (1977). Applications in plant pathology. In: Plant Tissues and Cell Culture. Eds: Street,H.E. 463-500. Blackwell, Oxford.

Inouye,M. (1979).What is the outer membrane? In: Bacterial Outer Membranes. Biogenesis and functions. Eds: Inouye,M. Wiley & Sons, London.

Israel,V., Rosen,H, & Evine,M. (1972). Binding of bacteriophage P22 tail parts to cells. Journal of Virology 10, 1152-1158.

Izard,C., Lacharpagne,J. & Schiltz,P. (1964). Behaviour of Perenospora tabacina in tissue cultures and role of the leaf epidermis. Annales de la Direction des Etudes et de l'Equipment 2, 95-99.

Jennings,D.L. (1960).Observations on virus diseases of cassava in resistant and susceptible varieties. I.Mosaic disease. Empire Journal of Experimental Agriculture 28, 23-34.

Jennings,D.L. (1970). Cassava in Africa. Field Crop Abstracts 23, 271-277.

Jennings,D.L. (1972). Breeding for resistance to cassava virus in East Africa. In: Proceedings IITA Cassava Mosaic workshop, IITA, Ibdan, Nigeria. 48pp.

Jennings,D.L. (1976). Cassava, Manihot esculenta (Euphorbiaceae). In: Evolution of Crop Plants Ed:N.W.Simmonds. 81-84pp. Longman, London.

Jones,S.B. & Fett,W.F. (1985). Fate of Xanthomonas campestris infiltrated into soybean leaves: an ultrastructural study. Phytopatholgy 75, 733-741.

Joseph,J. & Elango,F. (1991). The status of cassava bacterial blight caused by Xanthomonas campestris pv manihotis in Trinidad. Journal of Phytopathology 133, 320-326.

Kamoun,S., Cooley,M.B., Rogowsky,P.M. & Kado,C.I. (1989). Two chromosomal loci involved in production of loci involved in exopolysaccharide in Agrobacterium tumefaciens. Journal of Bacteriology 171, 1755-1759.

Karr,A.L. & Albersheim,P. (1970). Polysaccharide degrading enzymes are unable to attack plant cell walls without prior action by a 'wall modifying enzyme'. Plant Physiology 46, 69-80.

Kartha,K.K. & Gamborg,O.L. (1979). Cassava tissue culture- Principles and Applications. In: Plant Cell and Tissue Culture: Principles and Applications. Eds: Sharp,W.R., Larson,P.O., Paddock,E.F. & Raghaven,V. 711-725pp. Ohio State University Press, Colombus.

Kartha,K.K. & Gamborg,O.L., Constabel,F. & Shyluk,J.P. (1974). Regeneration of cassava plants from apical meristems. Plant Science Letters 2, 107-113.

Keen,N.T., Williams,P.H. & Walker,J.C. (1967). Protease of Pseudomonas solanacearum in relation to cucumber angular leaf spot. Phytopatholgy 57, 263-271.

Kelman,A. & Cowlings,E.B. (1965). Cellulase of Pseudomonas solanacearum in relation to pathogenesis. Phytopatholgy 55, 148-155.

Keon,J.P.R. (1985). Cytological damage and cell wall modification in cultured apple cells following exposure to pectin lyase from Monilinia fructigena. Physiological Plant Pathology 26, 11-29.

Klement,Z. & Lovrekovich,L. (1961). Studies on host-parasite relations in beanpods infected with bacteria. Phytopathologische Zeitschrift 45, 217pp.

Knoche,H.W. & Duvick,J.P. (1987). The role of toxins in plant disease. In: Fungal Infection of Plants. Eds: Pegg,G.F. & Ayres,P.G. 158-192pp. Cambridge University Press. Cambridge.

Kotoujansky,A., Diolez,A., Baccara,V., Bertheau,Y., Andro,T. & Coleno, A. (1985). Molecular cloning of Erwinia chrysanthemi pectinase and cellulase structural genes. EMBO Journal 4, 781-785.

Kotoujansky,A. (1987). Molecular genetics of pathogenesis by soft rot erwinias. Annual Review of Phytopathology 25, 405-430.

Krishnamurthy, M.S. (1976). Physiology of host-parasite relationship with special reference to respiratory changes in cotton bacterial blight. Ph.D thesis, Medurai Kamaraj University, Madurai.

Kuo,M.S. & Scheffer,R.P. (1964). Evaluation of fusaric acid as a factor in development of Fusarium wilt. Phytopathology 54, 1041-44.

Lakshmanan,M. & Krishnamurthy,M.S. (1979). Respiratory changes in bacterial blight of cotton. Current Trends of Life Science 6, 437-46.

Lakshmanan,M. & Kathirvel Pandian,S. (1988). A new phytotoxin from Xanthomonas campestris pv malvacearum. Advances in Research on Plant Pathogenic bacteria 221-35.

Lamport,D.T.A. (1965). The protein component of primary cell walls. Advances in Botanical Research 2, 151-218.

Lange,E. & Knosel,D. (1970). Zur Bedeutung pectolytischer cellulolytischer und proteolytischer enzyme fur die virulenz phytopathogener bakteriun. Phytopathologische Zeitschrift 69, 315-329.

Larkin,P.J. & Scowcroft,W.R. (1981). Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theoritical and Applied Genetics 60, 197-214.

Leigh,J.A., Signer,E.R. & Walker,G.C. (1985). Exopolysaccharide-deficient mutants of Rhizobium meliloti that form ineffective nodules. Proceedings of National Academy of Science, U.S.A. 82, 6231-6235.

Leon,J. (1977). Origin, evolution and early dispersal of root and tuber crops. In: Proceedings of the fourth symposium of the International Society for Tropical Root Crops Eds: Cock,J., McIntyre,R. & Graham,M. 20-36pp. International Development Research Centre, Canada.

Leslie,K.A. (1987). The significance of root crops in the tropics. In: Proceedings of the International Symposium on Tropical Root Crops 5, 1-12.

Lewis,S. & Goodman,R.N. (1965). Mode of penetration and movement of fire blight bacteria in apple leaf and stem tissue. Phytopathology 55, 719-723.

Liao,C.H. & Wells,J.M. (1987). Association of pectolytic strains of Xanthomonas campestris with soft rots of fruits and vegetables at retail markets. Phytopathology 77, 418-422.

Liao,C.H. (1989). Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. Applied and Environmental Microbiology 55(7), 1677-1683.

Lindberg,A.A. (1977). Bacterial surface carbohydrates and bacteriophage adsorption. In: surface carbohydrates of the prokaryotic cell. Eds: Sutherland,I.W. 289-356pp. Academic Press, New York.

Lindberg,A.A., Wollin,R., Gemski,P. & Wohlhieter,J.A. (1978). Interaction between bacteriophage sf6 and Shigella flexneri. Journal of Virology 27, 38-44.

Lindsey,S.S., Wheeler,B., Sanderson,K.E., Costerton,J.W. & Chen,K.j. (1973). The release of alkaline phosphatase and of lipopolysaccharides during the growth of rough and smooth strians of Salmonella typhimurium. Canadian Journal of Microbiology 19, 335-343.

Loegering,W.Q. & Geiss, I.R. (1957). Independence in the action of 3 genes conditioning stem rust resistance in Red Egyptian wheat. Phytopathology 47, 740-741.

Lozano,J.C. (1972). Cassava bacterial blight in Colombia. Ph.D. Thesis. Unuiversity of Wisconsin. 115 pp.

Lozano,J.C. (1975). Bacterila blight of cassava. PANS 21, 38-43.

Lozano,J.C. (1986). Cassava bacterial blight: a manageable disease. Plant Disease 70(7-12), 1089-93.

Lozano,J.C. & Booth,R.H. (1974). Diseases of cassava (Manihot esculenta Crantz). PANS 20, 30-54.

Lozano,J.C. & Laberry,R. (1982). Screening for resistance to cassava bacterila blight. Plant Disease 66, 316-318.

Lozano,J.C. & Sequeira,L. (1974a). Bacterial blight of cassava in Colombia: Etiology. Phytopathology 64(1), 74-82.

Lozano,J.C. & Sequeira,L. (1974b). Bacterial blight of cassava in Colombia: Epidemiology and control. Phytopathology 64(1), 83-88.

Ludwig,R.A., Clark,R.V., Julien,J.B. & Robinson,D.B. (1956).Studies on the seedling disease of barley caused by Helminthosporium sativum P.K. & B. Canadian Journal of Botany 34, 653-73.

Luke,H.H. & Wheeler,H.E. (1955). Toxin production by Helminthosporium victoriae. Phytopathology 45, 453-458.

Lukens,J.H., Mathews,D.E. & Durbin,R.D. (1987). Effect of tagetittoxin on levels of ribulose 1,5-bisphosphate carboxylase, ribosomes and RNA in plastids of wheat leaves. Plant Physiology 84, 808-813.

Lyon,F. & Wood,R.K.S. (1976). The hypersensitive reaction and other responses of bean leaves to bacteria. Annals of Botany 40, 479-492.

MacDonald,W.L. & McNabb,H.S. (1974). Electron microscope observations of Ceratocystis ulmi induced tyloses development in Ulmus. European Journal of Forest Pathology 4, 2-10.

Magasanik, B. (1961). Catabolite repression. Cold Harbour Symposium on Quantitative Biology 26, 249-256.

Mandels,M. & Reese,E.T. (1960). Induction of cellulase in fungi cellobiose. Journal of Bacteriology 79, 816-826.

Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, New York.

Mansfield,J., Brown,I. & Maroofi,A. (in press). Bacterial pathogenicity and the plants response: ultrastructural, biochemical and physiological perspectives. In: Bacterial pathogenesis and disease resistance, fourth International Symposium on Biotechnology and Plant Bacteria. Eds: Bills,D.D.

Maraite,H. & Meyer,J.A. (1975). Xanthomonas manihotis (Arthaud- Berthet) Starr, causal agent of bacterial wilt, blight and leaf spots of cassava in Zaire. PANS 21(1), 26-37.

Marcus,H. & Baker,N.R. (1985). Quantitation of adherence of mucoid and nomucoid Pseudomonas aeruginosa to hamster tracheal epithelium. Infection and Immunity 47, 723-729.

Marre,E. (1979). Fusicoccin: a tool in plant physiology. Annual Review of Plant Physiology 30, 273-288.

Martin,F.W. (1976). Cytogenetics and plant breeding of cassava -a review. Plant Breeders Association 46, 909-916.

Matthysse,A.G., Wyman,P.M. & Holmes,K.V. (1978). Plasmid dependent attachment of Agrobacterium tumefaciens to plant tissue culture cells. Infection and Immunity 22, 516-522.

Matthysse,A.G. (1983a).The use of tissue cultures in the study of crown gall and other bacterial diseases. In: Use of tissue culture and protoplasts in Plant Pathology Eds: Helgeson,J.P. & Deverall,B. 39-68pp. Academic Press, Sydney.

Matthysse,A.G. (1987). A method for the bacterial elicitation of a hypersensitive-like response in plant cell cultures. Journal of Microbiological Methods 7, 183-191.

Mazau,D. & Tugaye,M.T. (1986). Hydroxy-proline rich glycoprotein accmulation in the cell walls of plants infected by various pathogens. Physilogical and Molecular Plant Pathology 29, 147-157.

Meyer,R.W. (1967). Tyloses development in white oak. Forest Products Journal 17, 50-56.

Meyer,R.W. & Cote,W.A. (1968). Formation of the protective layer and its role in tylosis development. Wood Science Technology 2, 84-94.

Miller,J.H. (1972). Experiments in molecular genetics. Cold Spring Harbour Laboratory, New York.

Mitchell,R.E. & Bieleski,K.L. (1977). Involvement of phaseolotoxin in halo blight of beans.Transport and conversion to functional toxin. Plant Physiology 60, 723-29.

Mitchell,R.E. (1978). Halo blight of beans: toxin production by several Pseudomonas phaseolicola isolates. Physiological Plant Pathology 13, 37-49.

Mitchell,R.E. (1981). Structure: bacterial. In: Toxins in Plant Disease Ed: Durbin,R.D. 259-293. Academic Press, New York.

Mitchell,R.E. (1984). The relevence of non-host -specific toxins in the expression of virulence by pathogens. Annual Review of Phytopathology 22, 215-245.

Mollenhaeur,H.H. & Hopkins,D.L. (1976). Xylem morphology of Pierce's disease-infected grapevines with different levels of tolerance. Physiological Plant Pathology 9, 95-100.

Moore,S. & Stein,W.H. (1958). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. Journal of Biological Chemistry 211, 907-913.

Morel,G. (1948). Recherches sur la culture associee de parasits obligatoires et de tissus vegetaux. Annales des Ephiphyties 14, 1-12.

Movahedi,S. (1990). Mechanisms of disease resistance to Botrytis cinerea in cold stored Daucus carota L. 95-96pp. PhD. thesis, University of London.

Mueller,W.C. & Beckman,C.H. (1984). Ultrastructure of the cell wall of vessel contact cells in the xylem of tomato cells. Annals of Botany 53, 107-114.

Muhlradt,P.F. & Golecki,R. (1975). Assymetrical distribution and artifactual re-orientation of LPS in the outer membrane bilayer of Salmonella typhimurium. Europian Journal of Biochemistry 51, 343-352.

Murashige,T. & Skoog,F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Nachmias,A., Orenstein,J., Tal,M. & Goren,M. (1990). Reactions to a Verticillium dahliae phytotoxin in tissue cultures derived from susceptible and tolerant potato. Plant Science 68, 123-130.

Nagel,C.W. & Anderson,M.M. (1965). Action of bacterila trans-eliminase on normal and unsaturated oligogalcturonic acids by an endo-pectic acid trans-eliminase. Archives of Biochemistry and Biophysics 118, 590-595.

Nasuno,S. & Starr,M.P. (1967). Polygalacturonic acid trans-eliminase of Xanthomonas campestris. Biochemical Journal 104, 178-185.

Neilands,J.B. (1982). Microbial envelope proteins related to iron. Annual Review of Microbilogy 36, 285-309.

Neilands,J.B. (1984). Siderophores of bacteria and fungi. Microbiological Sciences 1, 9-14.

Nelson,P.E. & Dickey,R.S. (1970). Histopathology of plants infected with vascular bacterial pathogens. Annual Review of Phytopathology 8, 259-280.

Nemec,S. (1975). Vessel blockage by myelin forms in citrus with and without rough-lemon decline symptoms. Canadian Journal of Botany 53, 102-108.

Nishiyama,K., Sakai,R., Ezuka,A., Ichihara,A., Shiraishi,K. & Sakamura,S. (1977). Detection of coronatine in halo blight of Italian rye grass. Annals of Phytopathological Society of Japan 43, 219-220.

Nolt,B.L., Pineda B. & Velasco,A.C. (1992). Surveys of cassava plantations in Colombia for virus and virus-like diseases. Plant Pathology 41, 348-354.

Norkrans,B. (1980). Surface microlayers in aquatic environments. In: Advances in Microbial Ecology Eds: Alexander,M. 51-86pp. Plenum Press, New York.

Normanha,E.S. (1971). Yuca: observaciones y recommendaziones sobre su cultivo en Nicaragua. Managua, Banco Central de Nicaragua, 29 PP.

Nossal,N.G. & Heppel,L.A. (1966). The release of enzymes by osmotic shock from E. coli in exponential phase. Journal of Biological Chemistry 241, 3055.

O'Brien,I.G. & Gibson,F. (1970). The structure of enterochelin and related 2,3-dihydroxy-N-benzoyl-serine conjugates from E. coli. Biochemica et Biophysica Acta 215, 393-402.

Odvody,G.N., Dunkle,L.D. & Edmunds,L.K. (1977). Characterization of the Periconia circinata population in a Milo disease nursery. Phytopathology 67, 1485-1489.

Ohman,D.E. (1986). Molecular genetics of exopolysaccharide production by mucoid Pseudomonas aeruginosa. Europian Journal of Clinical Microbiology 5, 6-10.

Oulette,G.B. (1980). Occurrence of tyloses and their ultrastructure differentiation from similarly configured structures in American elm infected by Ceratocystis ulmi. Canadian Journal of Botany 58, 1056-1073.

Onwueme,I.C. (1978). The tropical tuber crops. 109-163.John Wiley and Sons.

Orieux,L. & Felix,S. (1968). List of plant diseases in Mauritius. CMI series Phytopathological papers 7, 38

Owens,L.D. & Wright,D.A. (1965). Rhizobial induced chlorosis in soybeans: Isolation, production in nodules and varietal specificity of the toxin. Plant Physiology 40, 927-30.

Oyenuga,V.A. (1968). Nigeria's foods and feeding stuff, Ibdan University Press, Nigeria. 99 pp.

Palmieri,M.R., Tomasello,D. Lio,D. (1979). Originee caratterizzazione istochimica delle gomme in piante di agrumi con sindromi a diversa eziologia. Rivista di Patalogia Vegetale S IV 15, 43-49.

Panopoulas,N.J. & Peet,R.C. (1985). The molecular genetics of plant pathogenic bacteria and their plasmids. Annual Review of Phytopathology 23, 381-419.

Panopoulas,N.J. & Staskawicz,B.J. (1981). Genetic of production. In: Toxins in PLant Disease Ed: Durbin R.D. 79-107pp. Academic Press, New York.

Patel,P.N. & Walker,J.C.(1963). Changes in free amino acid and amide content of resistant and susceptible beans after infection with the halo blight organism. Phytopathology 53, 522-28.

Patil,S.S., Tam,L.W. & Kolattukudy,P.E. (1972). Isolation and the mode of action of the toxin from Pseudomonas phaseolicola. In: Phytotoxins in Plant Diseases. Eds:Wood,R.K.S., Ballio, A.& Graniti, A. 530pp. Academic Press, London.

Patil,S.S. (1974). Toxins produced by phytopathogenic bacteria. Annual Review of Phytopathology 12, 259-79.

Patil,S.S., Hayward,A.C. & Emmons,R. (1974). An ultra-violet induced non-toxigenic mutant of Pseudomonas phaseolicola of altered pathogenicity. Phytopathology 64, 59-95.

Pegg,G.F. (1981a). The involvement of growth regulators in the diseased plant. In:Effects of disease on the physiology of the growing plant. Eds: Ayres,P.G. 149-177. Cambridge University Press.

Pegg,G.F. (1981b). The role of growth regulators in plant disease. In: Plant diseases: infection, damage and loss. Eds: Wood,R.K.S. & Jellis,G.J. 29-48pp. Blackwell, Oxford.

Pegg,G.F. (1989). Pathogenesis in vascular diseases in plants. In: Vascular wilt diseases of plants Eds: Ramos,E.C. & Beckman,C. 51-94pp. Springer Verlag, Berlin.

Pegg,G.F., Gull,K. & Newson,R.J. (1976). Transmission electron microscopy of Verticillium albo-atrum hyphae in xylem vessels of tomato plants. Physiological Plant Pathology 8, 221-224.

Pereira,A.L.G. & Zagatto,A.G. (1967). Etiologia da 'mancha angular' na folha da mandioca (Manihot utilissima). Archivos Instituto Biologico, Sao Paulo 34(3), 153-160.

Perreaux,D. (1979). Study of toxin production by Xanthomonas manihotis. In: Diseases of Tropical Food Crops, Proceedings of an International Symposium Eds:H.Maraite & J.Meyer. 161-67pp.

Perreaux,D., Maraite,H. & Meyer,J.A. (1978). Histopathological study by fluorescent microscopy of cassava stems infected by Xanthomonas manihotis. Proceedings of the fourth International Conference in Plant Pathogenic Bacteria. Angers.

Perreaux,D., Maraite,H. & Meyer,J.A. (1982). Identification of 3(methylthio) propionic acid as a blight inducing toxin produced by Xanthomonas campestris pv manihotis in vitro. Physiological Plant Pathology 20, 313-19.

Perreaux,D., Maraite,H. & Meyer,J.A. (1986). Detection of 3(methylthio) propionic acid in cassava leaves infected by Xanthomonas campestris pv manihotis. Physiological and Molecular Plant Pathology 28, 323-28.

Perry,B.A. (1943). Chromosome number and phylogenetic relationships in the Euphorbiaceae. American Journal of Botany 30, 572-543.

Persley,G.J. (1976). Distribution and importance of cassava bacterial blight in Africa. In: Cassava bacterial blight- Report of an interdisciplinary workshop held at IITA, Ibdan, Nigeria Eds: G.Persley, E.R.Terry & R. MacIntyre. 9-14pp.

Pijut,P.M., Domir,S.C., Lineberger,R.D. & Schreiber,L.R. (1990). Use of culture filtrates of Ceratocystis ulmi as a bioassay to screen for disease tolerant Ulmus americana. Plant Science 70, 191-196.

Pinkerton,F. & Strobel,G. (1976). Serinol as an activator of toxin production in attenuated cultures of Helminthosporium sacchari. Prtoceedings of the National Academy of Science. 73, 4007-4011.

Pollack,J.R. & Neilands,J.B. (1970). Enterobactin and ion transport compounds from Salmonella typhimurium. Biochemical and Biophysical Research Communications 38, 989-992.

Powell,P.W. (1972). The Cercospora leaf spots of cassava. Tropical Root and Tuber Crops Newsletter 6, 10-14.

Pringle,R.B. & Scheffer,R.P. (1964). Host specific plant toxins. Annual Review of Phytopathology 2, 133-56.

Pupillo,P., Mazzucchi,U. & Pierini,G. (1976). Pectate lyase produced by Erwinia chrysanthemi Burkh. et al. in polypectate broth or in Dieffenbachia leaves. Physiological Plant Pathology 9, 113-120.

Ramaley,R.F. (1979). Molecular biology of extracellular enzymes. Advances in Applied Microbiology 25, 27-35.

Ramirez,M.E., Fucikovsky,L., Jimenez,F., Quintero,R. & Galindo,E. (1988). Xanthan gum production by altered pathogenicity variants of Xanthomonas campestris. Applied Microbiological Techniques 29, 5-10.

Ramphal,R. & Pier,G.B. (1985). Role of Pseudomonas aeruginosa mucoid exopolysaccharides in adherence to tracheal cells. Infection and Immunity 47, 1-4.

Ray,T.C,, Smith,A.R.W., Carter,K.J. & Hignett,R.C. (1980). Composition of lipopolysaccharides from four strains of Erwinia amylovora. Journal of General Microbiology 132, 720-726.

Reddy,M.N., Stuteville,D.L. & Sorenson,E.L. (1971). Protease production during pathogenesis of bacterila leaf spot of alfalfa and by Xanthomonas alfalfae in vitro. Phytopathology 61, 361-365.

Reed,C.A. (1977). Origins of Agriculture. . Ed: Sol Tax.816-17pp. Monton, Paris.

Reynolds,E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17, 208-212.

Reid,J.L. & Collmer,A. (1984). A rapid, sensitive, high resolution activity stain for detecting and differentiating pectic enzymes in gels. Phytopathology 74, 834 (abstr.)

Reid,J.L. & Collmer,A. (1985). Activity stain for rapid characterization of pectic enzymes in iso electric focusing and sodium dodecyl sulfate-polyacrylamide gels. Applied and Environmental Microbiology 50(3), 616-622.

Rhodehamel,N. & Durbin,R.D. (1989). toxin production by strains of Pseudomonas syringae pv tagetis. Physiological and Molecular Plant Pathology 35, 301-11.

Roach,P.M. & Garnett,H.M. (1986). Suitability of cell suspension cultures for studing the interaction of cassava with the pathogen causing bacterial blight. South African Journal of Science 82, 392-394.

Robb,J., Brisson,J.D., Bush,L. & Lu,B.C. (1978). Ultrastructure of wilt syndrome caused by Verticillium dahliae V. Attempted localisation of phenolic compounds in the vascular region. Canadian Journal of Botany 56, 2594-2612..

Robb,J., Brisson,J.D., Bush,L. & Lu,B.C. (1979). Ultrastructure of wilt syndrome caused by Verticillium dahliae VII. Correlated light and transmission electron microscope identification of vessel coating and tyloses. Canadian Journal of Botany 57, 822-834.

Robb,J., Bush,L. & Rauser,W.E. (1980). Zinc toxicity and xylem vessel alterations in white beans. Annals of Botany 46, 43-50.

Robb,J., Bush,L (1982). Ultrastructural changes in drought induced wilt: a comparison with pathogen-induced flaccidity. Canadian Journal of Botany 4, 97-105.

Robeson,D.J. & Cook,D.R. (1985). Production of low molecular weight carboxylic acids by Xanthomonas campestris pv campestris in relation to the amino acid composition of the medium and their possible involvement in pathogenesis. Physiological Plant Pathology 26, 219-230.

Robinson,J.N. & Callow,J.A. (1986). Multiplication and spread of pathovars of Xanthomonas campestris in host and non-host plants. Plant Pathology 35, 169-177.

Roeder,D.L. & Collmer,A. (1985). Marker exchange mutagenensis of apectate lyase isozyme genein Erwinia chrysanthemi. Journal of Bacteriology 164, 51-56.

Rogers,D.J. (1963). Studies of Manihot esculenta Crantz (Cassava) and related species. Bulletin of Torrey Botany 90, 43-54.

Romeiro,R., Karr,A. & Goodman,R. (1981). Isolation of a factor from apple that agglutinates Erwinia amylovora. 68, 772-777.

Rossel,G., De Bertoldi,F.G. & Tizio,R. (1987). In vitro mass tuberisation as a contribution to potato micropropagation. Potato Research 30, 111-116.

Rott,P. & Chagvardieff,P. (1987). In vitro reactions of sugar cane (Saccharum sp.) plantlets inoculated with 2 strains of Xanthomonas albilineans (Ashby) Dowson. Plant Cell, Tissue and Organ Culture 9, 9-18.

Rudolph,K. (1976). Non-specific toxins. In: Encyclopedia of Plant Pathology 4, 235-70. Eds: R. Heitefuss & P.H. Williams. Springer Verlag, New York.

Rudolph,K.W.E., Gross,M. Neugebauer,M., Hokawat,S., Zachowski,A., Wydra,K. & Klement,Z. (1989). Extracellular polysaccharides as determinants of leaf spot diseases caused by xanthomonads and pseudomonads. In: Phytotoxins and Plant Pathogenesis Eds: Graniti,A., Durbin,R.D. & Ballio,A. 177-218pp. Springer-Verlag, Berlin.

Sabet,K.A. & Dowson,W.J. (1951). Action of phytopathogenic bacteria on pectate gel. Nature 23, 168-175.

Sachs,T. & Thimann,K.V. (1967). The role of auxins and cytokinins in the release of buds from dominance. American Journal of Botany 54, 136-144.

Sacristan,M.D. (1982). Resistance responses to Phoma lingum of plants regenerated from selected cell and embryogenic cultures of haploid Brassica napus. Theoritical and Applied Genetics 61, 193-200.

Scheffer,R.P. & Pringle,R.B. (1961). A selective toxin produced by Periconia circinata. Nature 191, 912-913.

Scheffer,R.P., Nelson,R.R. & Ullstrup,A.J. (1967). Inheritance of toxin production and pathogenicity in Cochliobolus carbonum and Cochliobolus victoriae. Phytopathology 57, 1288-1291.

Schultz,J.S. & Gerhardt,P. (1969). Dailysis culture of microorganisms.Theory and results. Bacteriological Reviews 33, 1-47.

Scolander,P.F., Hammel,H.T., Bradstreet,E.D. & Hemmingsen,E.A. (1965). Sap pressure in vascular plants. Science 148, 339-346.

Schnathorst,W.C. & DeVay,J.E. (1964). Virulence of Pseudomonas syringae in Gossypium hirsutum related to toxin production. Phytopathology 54: 1436 (abstr.).

Schwarz,B, (1926). Slijm Ziekte in de cassva, Indische culturen(Teysmania)xi 17, 498-499.

Scowcroft,W.R., Larkin,P.J. & Brettel,R.I.S. (1983). Genetic variation from tissue culture. In: Use of Tissue Culture and Protoplasts in Plant Pathology Eds: Helgeson,J.P. & Deverall,B.J. 139-162pp. Academic Press, Sydney.

Sequeira,L., Garrd,G. & De Zoeten,G.A. (1977). Attachmnet of bacteria to host cell walls: its relation to mechanisms of induced resistance. Physiological Plant Pathology 10, 43-50.

Sequeira,L. & Graham,T.L. (1977). Agglutination of avirulent strains of Pseudomonas solanacearum by potato lectin. Physiological Plant Pathology 11, 43-54.

Shahin,E.A. & Shepard, J.F. (1980). Cassava mesophyll protoplasts: isolation, proliferation and shoot formation. Plant Science Letters 17, 459-465.

Shaw,M. & Colotelo,N. (1961). The physiology of host-parasite relations VII. The effect of stem rust on the nitrogen and amino acids in wheat leaves. Canadian Journal of Botany 39, 1351-1372.

Shepard,D.V. & Pitt,D. (1976). Purification of phospolipase from Botrytis and its effects on plant tissues. Phytochemistry 15 1465-1470.

Shi,J., Mueller,W.C. & Beckman,C.H. (1991). Ultrastructural responses of vessel contact cells in cotton plants resistant or susceptible to infection by Fusarium oxysporum f. sp. vasinfectum. Phsiological and Molecular Plant Pathology 38, 211-222.

Sinden,S.L. & DeVay,J.E. (1967). The nature of the wide spectrum antibiotic produced by pathogenic strains of Pseudomonas syringae and its role in the bacterial canker disease of Prunus persicae. Phytopathology 57, 102 (abstr.)

Sinden,S.L. & Durbin,R.D. (1968). Glutamine synthetase inhibition: possible modes of action of wildfire toxin from Pseudomonas tabaci. Nature 219, 379-80.

Sinden,S.L. & Durbin,R.D. (1970). A comparison of the chlorosis inducing toxin from Pseudomonas coronafaciens with wild fire toxin from Pseudomonas tabaci. Phytopathology 60, 360-64.

Singh,D. & Smalley,E.B. (1969). Changes in amino acid and sugar constituents of the xylem sap of American elm following inoculation with Ceratocystis ulmi. Phytopathology 59, 891-896.

Smidt,M. & Kosuge,T. (1978). The role of indole-3-acetic acid accumulation by alpha methyl tryptophane resistant mutants of Pseudomonas savastanoi in gall formation on oleanders. Physiological Plant Pathology 13, 203-214.

Smith,W.K. (1958). A survey of the production of pectic enzymes by plant pathogenic and other bacteria. Journal of General Microbiology 18, 33-41.

Smith,J.J. & Mansfield,J.W. (1982). Ultrastructure of interactions between pseudomonads and oat leaves. Physiological Plant Pathology 21, 259-266.

Somodi,G.C., Jones,J.B. & Scott,J.W. (1991). Populations of Xanthomonas campestris pv vesicatoria in lesions of susceptible and resistant tomato genotypes. Plant Disease 75, 357-360.

Spackman,D.H., Stein,W.H. & Moore,S. (1958). Automatic recording apparatus for the use in the chromatography of amino acids. Annals of Chemistry 30, 1190-1206.

Starr,M.P. & Nasuno,S. (1967). Pectolytic activity of phytopathogenic xanthomonads. Journal of General Microbiology 46, 425-434.

Stamp,J.A. (1984). In vitro regeneration studies with cassava (Manihot esculenta). Ph.D. thesis, University of Birmingham.

Stamp,J.A. & Henshaw,G.G. (1986). Adventitious regeneration in cassava. In: Plant Tissue Culture and its Agricutural Applications. Eds: Withers,L.A. & Alderson,P.G. Butterworths, London.

Staskawicz,B.J., Dahlbeck,D., Miller,J. & Damm,D. (1983). Molecular analysis of virulence genes in Pseudomonas solanacearum. In: Molecular Genetics of the Bacteria-Plant Interaction. Eds: Puhler,A. 345-352pp. Springer-Verlag, Heidelberg.

Staskawicz,B.J. & Panopoulos,N.J. (1979). A rapid and sensitive microbilogical assay for phaseolotoxin. Phytopathology 69, 663-666.

Stephen,J. & Pietrowski,R.A. (1986). Bacterial toxins. Second Edition. Aspects of Microbiology 2. Van Nostrand Reinhold, U.K.

Stewart,J.M. (1961). Diamino-β hydroxypimelic acid II. Configuration of the isomers. Journal of American Chemical Society 83, 435-439.

St.Leger,R.J., Durrands,P.K., Cooper,R.M. & Charnley,A.K. (1988). Regulation of production of proteolytic enzymes by the entomopathogenic fungus Metarhizium anisopliae. Archives of Microbiology 150, 413-416.

Stoddard,R.W. (1984). The biosynthesis of poysaccharides. 354pp. Macmillan, New York.

Stoessl,A. (1981). Structure and biogenetic relations: Fungal non host-specific. In: Toxins in Plant Disease. Ed: R.D. Durbin. 109-219pp. Academic Press, New York.

Strauss,A., Gelhardt,C. & King,P.J. (1980). Methods for selection of drug-resistant plant cell cultures. In: Tissue Culture Methods for Plant Pathologists Eds: Ingram,D.S. & Helgeson,J.P. 239-248pp. Blackwell, Oxford.

Strobel,G.A. (1977). Bacterial Phytotoxins. Annual Review of Microbiology 31, 205-224.

Suhayda,C.G. & Goodman,R.N. (1981). Early proliferation and migration of Erwinia amylovora and subsequent xylem occlusion. Phytopathology 71, 697-707.

Sutton,J.C. & Williams,P.H. (1970). Comparison of extracellular polysaccharides of Xanthomonas campestris from culture and from infected cabbage leaves. Canadian Journal of Botany 48, 645-651.

Suzuki,N. (1957). Bulletin of National Institute of Agricultural Sciience (Japan). Ser C,8, 69-130pp. In: Wheeler,H. & Luke. H.H. (1963). Microbial toxins in plant disease. Annual Review of Microbiology 17, 223-232.

Sydow,H.P. (1913). Enumeration of Philippine fungi, with notes and descriptions of new species. I.Micromycetes Philippine Journal of Science 2, 22-25.

Tamari,K. & Kaji,J. (1954). Bull. Fac. Agr. Niigata Univ. 5, 33-39. In: Wheeler,H. & Luke. H.H. (1963). Microbial toxins in plant disease. Annual Review of Microbiology 17, 223-232.

Tang,J.L., Gough,C.L., Barber,C.E., Dow,J.M. & Daniels,M.J. (1987). Molecular cloning of protease genes from Xanthomonas campestris pv campestris: expression in E.coli and role in pathogenicity. Molecular and General Genetics 210, 443-448.

Thimann,K.V. & Sachs,T. (1966). The role of cytokinins in the 'fasciation' disease caused by Corynebacterium fascians. American Journal of Botany 53, 731-739.

Thomas,M.D., Langston-Unkefer,P.J., Uchytil,T.F. & Durbin,R.D. (1983). Inhibition of glutamine synthetase from pea by tabtoxine-β-lactam. Plant Physiology 71, 912-915.

Thorne,L., Tansey,L & Pollock,T.J. (1987). Clustering of mutations blocking synthesis of xanthan gum by Xanthomonas campestris. Journal of Bacteriology 169, 3593-3600.

Tilquin,J.P. (1979). Plant regeneration from stem callus of cassava. Canadian journal of Botany 57, 1761-1763.

Tribe,H.T. (1955). Studies in the physioligy of parasitism XIX on the killing of plant cells by enzymes from Botrytic cinerea and Bacterium aroideae. Annals of Botany 19, 351-368.

Trisler,P. Gottesman,S. (1984). Ion transcriptional regulation of genes necessary for capsular polysaccharide synthesis in E. coli K-12. Journal of Bacteriology 169, 184-191.

Turner,T.G. (1981). Tabtoxin produced by Pseudomonas tabaci, decreases Nicotiana tabacum glutamine synthetase and causes accumulation of ammonia. Physiological Plant Pathology 19, 57-67.

Turner,P., Barber,C. & Daniels,M. (1984). Behaviour of the transposons Tn5 and Tn7 in Xanthomonas campestris pv campestris. Molecular and General Genetics 199, 338-343.

Turner,T.G. & Taha,R.R. (1984). Contribution of tabtoxin to the pathogenicity of Pseudomonas syringae pv. tabaci. Physiological Plant Pathology 25, 55-69.

Uchytil,T.F. & Durbin,R.D. (1980). Hydrolysis of tabtoxins by plant and bacterial enzymes. Experientia 36, 301-302.

Van Alfen,N.K., McMillan,B.D., Turner,V. & Hess,W.M. (1983). Role of pit membranes in the macromolecule induced wilt of plants. Plant Physiology 73, 1020-1023.

Van Alfen,N.K. (1982). Wilts: concepts and mechanisms. In: Phytopathogenic Prokaryotes,Vol.I. Eds: Mount,M.S. & Lacy,G.H. 459-473pp. Academic Press, New York.

Van de Goot,P. (1937). Ziekten on plagen der culturgervassen in Nederlandsh-indio in 1936. Meded. Inst. pl. Ziekt. batavia 89. 104 pp.

Vanderslice,R.W., Doherty,D.H., Capage,M.A., Betlach,M.R., Hassler,R.A., Henderson,N.M., Ryan Graniero,J. & Tecklenburg,M. (1989). Genetic engineering of polysaccharides in Xanthomonas campestris. In:Recent Developments in Industrial Polysaccharides: Biomedical and Biotechnical Advances. Eds: Crescenzi,V., Dea,I.C.M. & Stovola,S.S. 145-156. Gordon and Breach Science, New York.

Vander Molen,G.E., Beckman,C.H. & Rodehorst,E. (1977). Vascular gelation: A general response following infection. Physiological Plant Pathology 60, 641-646.

VanGijsegem,F., Toussaint,A. & Schoonejans,E. (1985). In vivo cloning of the pectate lyase and cellulase genes of Erwinia chrsanthemi. The EMBO Journal 4(3), 787-792.

Viegas,A.P. (1943). Alguns fungos de mandioca 1. Bragantia, 3, 1-19.

Vincens,F. (1915). Une maladie cryptogamiqu de Manihot glaziovii, arbre a caoutchouc du Ceara. Boletin de la Societe de la Pathologie Vegetale de France 2, 22-25.

Viseur,J. & Tapia,F.M. (1987). In vitro co-culture as a tool for the evaluation of fire blight resistance in pears and apples. Acta Horticulturae 217, 273-281.

Wainwright,S.H. & Nelson,P.E. (1972). Histopathology of Pelargonium spp. infected with Xanthomonas pelargonii. Phytopathology 62, 1337-1347.

Wakimoto,S. (1985). The effect of filamentous phage infection on the growth, ultrastructure and virulence of Xanthomonas campestris pv oryzae. In: Abstracts of the conference on the genus Xanthomonas. Fallen Leaf Lake, California, 1985.

Wallis,F.M. (1977). Ultrastructural histopathology of tomato plants infected with Corynebacterium michiganense. Physiological Plant Pathology 11, 333-342.

Wallis,F.M., Rijkenberg,F.H.J., Joubert,J.J. & Martin,M.M. (1973). Ultrastructural histopathology of cabbage leaves infected with Xanthomonas campestris. Physiological Plant Pathology 3, 371-379.

Wallis,F.M. & Truter,S.J. (1978). Histopathology of tomato plants infected with Pseudomonas solanacearum with emphasis on ultrastructure. Physiological Plant Pathology 13, 307-317.

Watanabe,M. (1963). Studies on the nutritional physiology of Xanthomonas oryzae (Uyeda et Ishiyama) Dowson.11. Effects of C & N sources on multiplication of bacteria. Annals of Phytopathological Society of Japan 28, 201-208.

Webb,K.J. & Gay,J.L. (1980). The recalcitrant powdery mildews- attempts to infect cultured tissues. In: Tissue Culture Methods for Plant Pathologists Eds: Ingram,D.S. & Helgeson,J.P. 151-159pp. Blackwell, Oxford.

Webster,D.M., Temple,S.R. & Galvez,G. (1983). Expression of resistance to Xanthomonas campestris pv phaseoli in Phaseolus vulgaris under tropical conditions. Plant Disease 67, 394-396.

Wiendling,R. (1948). Bacterial blight of cotton under conditions of artificial inoculation. U.S. Dept. of Agriculture Technology Bulletin No.956.

Wheeler,H.(1976). The role of phytotoxins in specificity. In: Specificity in Plant Diseases Eds: R.K.S.Wood & A.Graniti 217-35. Plenum Press, New York.

Wheeler,H. & Hanchey,P. (1968). Permeability phenomena in plant disease. Annual Review of Phytopathology 6, 331-350.

Wheeler,H. & Luke,H.H. (1963). Microbial toxins in plant disease. Annual Review of Microbiology 17, 223-232.

Whitfield,C., Sutherland,I.W. & Cripps,R.E. (1981). Surface polysaccharides in mutants of Xanthomonas campestris. Journal of General Microbiology 124, 385-392.

Wiehe,P.O. & Dowson,W.J. (1953). A bacterial disease of cassava (Manihot utilissima) in Nyasaland. Empire Journal of Experimental Agriculture 21(81), 141-143.

Williams,C.N. & Ghazali,S.M. (1969). Growth and productivity of tapioca (Manihot utilissima) 1. Leaf characteristics and yield. Experimental Argiculture 5, 183-194.

Williams,P.H., Reddy,M.N. & Strandberg,J.O. (1969). Growth of noninfected and Plasmodiophora brassicae infected cabage callus in culture. Canadian Journal of Botany 47, 1217-1221.

Williams,R.J., Agboola,S.D. & Schneider,R.W. (1973). Bacterial wilt of cassava in Nigeria. Plant Disease Reporter 57(10), 824-827.

Willmot,D.B., Nickell,C.D., Widholm,J.M. & Gray,L.E. (1989). Evaluation of soybean resistance to Philophora gregata culture filtrate in tissue culture. Theoritical and Applied Genetics 77, 227-232.

Wood,R.K.S.(1961). Verticillium wilt of tomatoes-the role of pectic and cellulolytic enzymes. Annals of Applied Biology 49, 120-139.

Wood,R.K.S. (1976). Specificity - an assessement. In: Specificity in Plant Diseases Eds: R.K.S.Wood & A.Graniti 327-38pp. Plenum Press, New York.

Wood,P.J. (1981). The use of dye-polysaccharide interactions in β-D glucanase assay. Carbohydrate Research 94, 19-23.

Xu,P., Iwata,M., Leong,S. & Sequeira,L. (1990). Highly virulent strains of Pseudomonas solanacearum that are defective in extracellular polysaccharide production. Journal of Bacteriology 172, 3946-3951.

Yoder,O.C. (1980). Toxins in pathogenesis. Annual Review of Phytopathology 18, 103-129.

Youle,D. & Cooper,R.M. (1987). Possible determinants of pathogenicity of Erwinia amylovora: evidence for an induced toxin. Acta Horticulturae 217, 161-166.

Young,J.M. (1973). Development of bacterial populations in vivo in relation to plant pathogenicity. New Zeland Journal of Agricultural Research 17, 105-113.

Zeigler,R.S., Powell,L.E. & Thurston,H.D. (1980). Gibberellin A4 production by Sphaceloma manihoticola. Phytopathology 70, 589-593.

Zimmerman,M.M. (1983). Pathology of the xylem. In: Xylem structure and the ascents of sap. 107-125pp. Springer-Verlag, New york.

Zucker,M. & Hankin,L. (1970). Physiological basis for a cycloheximide-induced soft rot of potatoes by Pseudomonas fluorescens. Canadian Journal of Micrbilogy 17, 1313-1318.

Zucker,M. & Hankin,L. & Sands,D. (1972). Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiological Plant Pathology 2, 59-67.

Zyngier de Resnik,F.C. (1968). Bacateriosis en Mandioca. Hoja informe INTA 26, 2.